FINAL TECHNICAL REPORT

NREL Subcontract XAC-4-13363-01
Reporting Period 31 October 1994—1 February 1997

Identification of Inhibitory Components in Dilute Acid Pretreated Lignocellulosic Materials

Thilini D. Ranatunga, Judith Jervis and Richard F. Helm
Fralin Biotechnology Center
Department of Wood Science and Forest Products
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061-0346

INTRODUCTION

Lignocellulosic materials from woody biomass have been targeted as a potential substrates for industrial ethanol production, and numerous studies have been carried out on bioconversion of woody biomass to fuel ethanol.[1-7] Most efforts in the United States have centered on the conversion of hardwoods, due to the large quantities of these species in the eastern portion of the United States. A pilot-plant study on bioconversion of woody biomass to fuel ethanol is currently being carried out at the National Renewable Energy Laboratory (NREL), and this technical report is part of this project. The NREL process is two-stage, and involves both chemical and enzymatic treatments. In the first stage, the biomass is exposed to dilute sulfuric acid at high temperatures, bringing about destruction of the hemicellulosic component. For hardwood and most agricultural biomass hemicelluloses, the predominant pretreatment reactions are deacetylation and depolymerization. This aqueous fraction is then subjected to fermentation with a recombinant *Zymomonas*. The cellulose and lignin remain relatively intact during the acid treatment and these solids are simultaneously saccharified and fermented in the second stage to provide ethanol and lignin.[8] The major products of the entire process are therefore ethanol and lignin.

The economical production of ethanol from wood necessitates the complete conversion of all available carbohydrates in the lignocellulosic material. The pentose sugar xylose is the major hydrolysis product of the hardwood hemicellulose termed glucuronoxylan (or simply xylan), and xylan constitutes 10-26% of the dry weight of temperate zone North American hardwoods.[9] Unfortunately xylose is a difficult sugar for most microorganisms to convert to ethanol, and for some organisms, the acetic acid formed during the acid treatment impedes the process. In addition, various other inhibitory compounds formed during the pretreatment process are toxic to these microorganisms.[7] Inhibition by any one compound could seriously jeopardize the economic success of wood-to-ethanol producing facilities. Thus, attention should be given

to development of improved methods for efficient conversion of all available sugars in the substrate before process can be implemented on the industrial scale.

The novel bacterium *Zymomonas mobilis* CP4 (pZB5) is a genetically modified bacterium capable of efficient production of ethanol from the pentose sugar xylose, as well as glucose.[10] The combined xylose and glucose fermenting capabilities of this organism makes it a potential biocatalyst for economical ethanol production. However, the ethanol fermentation efficiency of *Zymomonas* is substantially hindered by the toxic substances present in acid pretreatment hydrolyzate streams. Most of these toxic components are formed during the dilute acid pretreatment process.[11-14]

Under ideal conditions, the acidic treatment of xylan liberates xylose, acetic acid, and 4-O-methyl-D-glucuronic acid. However, these conditions are not achieved during industrial processing. Dehydration of xylose under acidic conditions forms furfural and hexose dehydration forms hydroxymethylfurfural (HMF).[15] In addition, lignin and extractives present in wood can be solubilized during the pretreatment process and/or undergo acid-catalyzed degradation reactions. Thus, in addition to the acetic acid formed from xylan breakdown, lignin, carbohydrate and extractive decomposition products can be formed in the hydrolyzate which can attenuate the efficiency of the bioconversion process. Ultimately, an overall decrease in the potential ethanol yield occurs making the process economically unattractive.

Identification and removal of inhibitory compounds should improve the efficiency of the biomass to ethanol conversion process. However, besides information on acetic acid and the furans, little quantitative information is known about the nature of these inhibitors. Thus, the major focus of this subcontract has been to obtain a firmer quantitative understanding of the role inhibitors play in hemicellulose hydrolyzate streams, and their effect on xylose to ethanol fermentation by the recombinant *Zymomonas mobilis* CP4(pZB5). With this information in hand, these studies led towards the evaluation of several detoxification strategies to improve the ethanol-from-biomass process.

RESULTS AND DISCUSSION

1. Analyses of Mixed Hardwood Hydrolyzate and Solid Biomass. Initial component analyses were carried out on a liquid hydrolyzate produced by NREL from a 1:1:1 mixture of red oak:white oak:yellow poplar, as well as the remaining solids, and untreated red oak, white oak, and yellow poplar sawdust. HPLC analyses of the liquid hydrolyzate using an HPX-87H analytical column indicated the major components to be glucose (2.4 ± 0.48 mg/mL), xylose (44.7±2.02 mg/mL), acetic acid (9.2 ± 0.43 mg/mL), and furfural (0.91 ± 0.051 mg/mL).[16] Once the concentration of the more important components of the hydrolyzate had been determined attempts were taken to separate the hydrolyzate. A solid phase extraction using a Diaion HP-

20 column failed due to the inability to recover furfural, which becomes irreversibly bound to the solid matrix.[16,17] The second fractionation attempt involved a MTBE (methyl *tert*-butyl ether) solvent extraction.[16,17] The HPLC analysis of the aqueous and MTBE fractions revealed that this extraction is helpful in removing most of the acetic acid and furans from the aqueous phase (which contains the carbohydrates). Reverse phase HPLC analyses of the MTBE-extracted material revealed the presence of furfural, HMF (0.068 ± 0.007 mg/mL), and gallic acid (0.17 ± 0.06 mg/mL). In addition, vanillin, syringaldehyde, protocatechuic acid, and sinapic acid were present in the hydrolyzate to a significantly lesser extent.[18] GC/MS analysis of the silylated MTBE extracts also supported the presence of these compounds.[16]

The solid biomass samples (untreated red oak, white oak, and yellow-poplar, and treated solids) were subjected to acetone:water and methanol extractions, and a summative biomass analysis was carried out on the extractive free biomass.[19] The yields from the acetone:water and methanol extractions were reported previously.[16] The summative biomass analysis of the extractive free biomass was carried out by a method adopted from Kaar and co-workers, and the results are given in Table I.

Table I. Summative Analysis of the Biomass Samples.

Component (%)	Red Oak	White Oak	Yellow-poplar	Treated Solids
Hexosan[a]	35.20	36.51	38.99	53.28
Pentosan[b]	16.70	17.70	14.53	0.02
Acid soluble lignin	3.14	3.49	3.48	1.14
Klason lignin	24.50	24.50	23.0	28.0
Ash	0.8	2.0	1.2	0.0
Moisture (105 °C)	7.99	7.46	7.59	8.99
Total[c]	88.33	91.66	88.79	91.43

[a]Corrected for mass change according to the eqn: Hexosan = (glucose)(0.9) + (HMF + 162/110). [b]Corrected for mass change according to the eqn: Pentosan = (xylose)(0.88) + (furfural + 132/96). [c]Total = % Hexosan + % Pentosan + % Klason lignin + % Acid-soluble lignin + % Ash + % Moisture. This total does not account for acetates, uronic acids or other hemicellulosic and pectic sugars.

A detailed description of the procedure has been reported,[17,19] and is essentially the same as the NREL LAP (Laboratory Approved Procedure) "Two-Stage Sulfuric Acid Hydrolysis for Determination of Carbohydrates" except for the correction factors used. The NREL LAP describes a correction for carbohydrate losses involving the submission of individual sugar standards to the two-stage hydrolysis and subsequently determining recovery. However, exposing the carbohydrates directly to the hydrolysis protocol is not the same as the exposure of fiber to the hydrolysis. The pure sugar samples when subjected to the 72% dilute sulfuric acid procedure, have a longer time to decompose (no depolymerization process). Thus, we feel

that NREL method is slightly overestimating the sugar values. In our method a correction was made based on the assumption that all carbohydrates destroyed during the acidolysis process are converted to either HMF or furfural. Thus, any decomposition of sugars should be quantified by the HMF and furfural values, and these values can be added to glucose and xylose values respectively. In addition, the hexosan and pentosan values are corrected for mass changes associated with hydrolysis. Although the assumption that the conversion of glucose and xylose to HMF and furfural is quantitative is an over-simplification, we feel that our conversion factor is a bit more accurate.

It should be pointed out however, the method employed to estimate the percent biomass does not account for the xylan acetate groups or uronosyl moieties nor does it account for the other hemicellulosic and pectic sugars. Thus the totals obtained were less than a 100%. The ash contents for the analyses are probably higher than the true values due to the high degree of error associated with the gravimetric determinations.

2. *Toxicity Studies.*

2a. Toxicity Studies on *Zymomonas mobilis* CP4(pZB5). Once the major components of the hydrolyzate was determined, the toxic effects of various fractions of the hydrolyzate as well as individual compounds present were tested toward *Zymomonas mobilis* xylose fermentation and *Saccharomyces cerevisiae* glucose fermentation.[17,20] In addition, several other potential inhibitory components believed to be present in liquid hydrolyzate streams were also tested. All the bioassays were performed according to NREL protocol.[21] The initial xylose and glucose concentrations in the bioassay experiments were adjusted to those of original liquid hydrolyzate, and the fermentation efficiencies were monitored by cell growth inhibition as well as determining ethanol yields after 48 or 72 h periods.[17,20] The inhibitor levels were tested at 100%, 200%, and 500% for all the compounds except for acetic acid. Higher than anticipated levels were tested to ensure toxic effects. Acetic acid was tested at 20%, 50%, and 100% level, and the ethanol yields were determined using HPLC. The OD values obtained with these potential inhibitors were reported previously.[17] Table II represents the ethanol yields obtained with *Zymomonas mobilis* for various hydrolyzate fractions and various individual compounds (at a 100% level).[22] During these experiments, the hydrolyzate testing had to be limited to a 30% level (v/v; 30 mL of hydrolyzate supplemented with sugars) due to severe precipitation observed at higher levels. Even at the 30% level the precipitation makes the OD method for ascertaining cell growth not very reliable. However, from the ethanol yields, it is obvious that the hydrolyzate inhibits the ability of the recombinant *Zymomonas* to ferment xylose to ethanol.[17] That the overlimed hydrolyzate (30% level) did not have a significantly improved fermentation efficiency indicates that overliming alone is not sufficient for removing toxic substances from the hydrolyzate.

Table II. Ethanol Fermentation Yields Obtained After a 48h Fermentation with Various Hydrolyzate Fractions and Individual Suspected *Zymomonas* Inhibitors (100% level).

Sample	Inhibitor Conc.[a] mg/mL	Ethanol[b] mg/mL	Percent Performance
Positive Control[c]	—	13.43-14.83	100
Negative Control[c] (30% loading)	—	1.37-1.43	9
Acetic acid	9.03	<1	0
Caproic acid	0.064	7.64	57
Furfural	0.95	8.52	58
Syringaldehyde	0.130	9.48	64
Vanillin	0.043	8.71	65
Protocatechuic acid	0.050	9.68	72
Glaucine	0.052	10.13	75
Gallic acid	0.173	11.39	77
HMF	0.090	11.86	80
Coniferyl alcohol	0.050	12.10	82
Sinapic acid	0.060	11.93	89
Syringic acid	0.093	12.80	95
Vanillic acid	0.084	13.63	101

[a]Inhibitor concentrations used in this study were estimated from the HPLC analyses of compounds detected in the hydrolyzate, or are based on the values from Ref. 11. The glaucine levels were estimated based upon extractive content of yellow-poplar, and assuming that glaucine was 10% of the extract. [b]Samples were added to the media dissolved in 0.1 mL of ethanol. Thus, the ethanol yields reported are subtracted from the initial ethanol in the media. [c]The positive control functions as a pure sugar control, and the initial sugars in the positive control represent the actual amounts in the original hydrolyzate. The negative control consists of 30%(v/v) overlimed hydrolyzate supplemented with sugars to represent those of the original hydrolyzate. The experiments were carried out in two setups, and the ethanol yield ranges reported for the two controls 13.43-14.83 and 1.37-1.43 corresponds to yields for positive and negative control, respectively for the two experiments.

The ethanol yields (Table II) obtained in the presence of individual potential toxic components indicated that acetic acid by far is the most toxic compound for *Zymomonas* followed by caproic acid and furfural. Except for vanillin and syringaldehyde, the other phenolic compounds tested had only a moderate effect. From this it is readily apparent that most of the compounds are only slightly inhibitory to cell growth and ethanol production, with organic acids being the strongest inhibitors. A trend is also observed where organic acids and aldehydes are more inhibitory than lignin acid, alcohols or the one alkaloid studied (glaucine). These results suggest that efforts at minimizing toxicity should be initially directed at removing the acetic acid formed during the acid hydrolysis.

Toxicity testing was also carried out on the extracts from untreated red oak, white oak, yellow poplar, and the treated mixed hardwood solids.[22] As mentioned previously, lignin and extractives present in woody biomass can be solubilized during the acid pretreatment process. These compounds are potential inhibitors of ethanologenic microorganisms. Thus it was important to ascertain the toxicity of these fractions, as species with highly toxic extracts may not be desirable for industrial bioconversion. The acetone:water (7:3) and methanol extracts of these solid samples were prepared according to the previously described method,[16] and the toxic effects of these freeze-dried extracts were tested against the recombinant *Zymomonas*. The ethanol yields were determined using a GC method,[22] and the results are given in Table III. The ethanol yields indicate that the red oak and white oak acetone:water extracts are more toxic toward *Zymomonas* than the methanol extracts. In both instances, white oak extractives were observed to be the most toxic, and yellow poplar to be the least toxic.

Table III. Ethanol Fermentation Yields after 48 h for the Controls and Wood Extracts.

Sample (% Loading)[a]	Ethanol (mg/mL)[b] Acetone: Water extract (% Performance)		Ethanol (mg/mL)[b] Hot Methanol Extract (% Performance)	
Positive Control	16.79	(100)	16.79	(100)
Negative Control	1.12	(7)	1.12	(7)
Red oak (30%)	1.26	(8)	7.23	(43)
Red oak (15%)	1.40	(9)	12.47	(74)
Red oak (5%)	6.81	(41)	11.87	(71)
White oak (30%)	<1.0	(0)	<1.0	(0)
White oak (15%)	1.32	(8)	<1.0	(0)
White oak (5%)	4.71	(28)	3.51	(21)
Yellow poplar (30%)	11.26	(67)	11.33	(67)
Yellow poplar (15%)	13.72	(82)	12.87	(77)
Yellow poplar (5%)	11.86	(70)	12.43	(74)
Treated solids (30%)	2.82	(17)	13.76	(82)
Treated solids (15%)	10.03	(60)	14.60	(87)
Treated solids (5%)	13.59	(81)	12.02	(72)

[a]The levels of extractives added to the media were estimated based on the relationship between extractive yields and the amount of xylose found in the wood hydrolyzate:

(% yield of extract)/(% xylose in wood) = (extract conc. in hydrolyzate)/(xylose conc. in hydrolyzate) (see Ref. 22 for a sample calculation). This represents the relationship between extractive and xylose contents if one assumes that all of the extractives and xylose were solubilized by the pretreatment process. [b]All the extracts were freeze-dried to ensure complete removal of solvents, and the extractives were added to the media dissolved in ethanol:water (1:1, 0.2 mL). Ethanol values reported are values obtained after subtracting initial ethanol in media (at T_0).

These results correlate with what is known about wood and its resistance to microbial decay. Of the three species, the White oaks are considered to be the most durable followed by the Red oaks and Yellow-poplar.[23,24] This resistance to decay is thought to be mostly due to the tannins, which are present in significantly higher quantities in the oaks than in yellow-poplar. Although the mechanism of biological resistance is not completely clear, tannins are well known for their ability to precipitate proteins. Extracellular degradatory enzymes secreted by a wood-invading organism would, when in intimate contact with a tannin molecule, undergo precipitation and subsequent inactivation. The oak extractives being much more inhibitory than yellow-poplar suggests that yellow-poplar or fast growing hardwood species such as cottonwood, aspen or poplar (low tannin content woods) would be much more desirable substrates for bioconversion than the oaks.

Removal of compounds from wood hydrolyzates that are inhibitory to microorganisms should improve the overall efficiency of the biomass conversion process. The results obtained here suggest that with respect to xylose utilization, the increase in fermentation is low, as long as significant quantities of acetic acid remain. A qualitative ranking was established for potential inhibitory compounds and extracts (Figure 1), and the ranking indicates that acetic acid and oak extracts are the most inhibitory compounds present in the hydrolyzate, with furfural exhibiting a moderate effect. Solvent extraction method such as a MTBE extraction may be useful in separating a fairly toxic organic fraction from the liquid hydrolyzate. From the above studies, a manuscript entitled *Identification of Inhibitory Components Toxic Toward* Zymomonas mobilis *CP4(pZB5) Xylose Fermentation* was prepared, and submitted for publication in *Applied Biochemistry and Biotechnology*. A revised, accepted manuscript is attached as Appendix B.

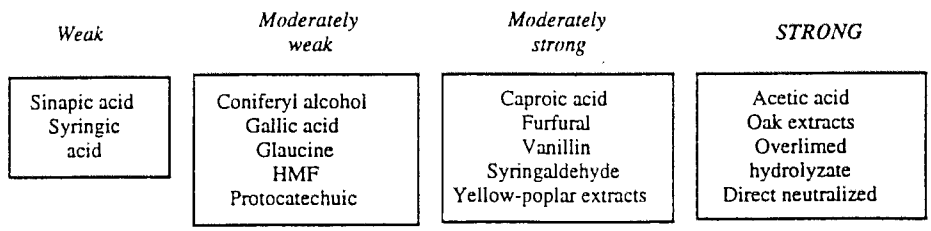

Figure 1. Relative Compound/Extract Inhibition Scale for *Zymomonas mobilis* CP4(pZB5) Xylose Fermentation.

2b. Toxicity Testing of *Saccharomyces cerevisiae* D5A. The toxic effects of various hydrolyzate fractions, potential inhibitory compounds and extracts from solid biomass were tested with the glucose fermenting yeast, *Saccharomyces cerevisiae* D5A.[20] All the bioassay experiments were carried out according to NREL protocols, and the results from these studies have been reported in detail.[20] The glucose fermentation efficiency of this organism was significantly inhibited by a 60% hydrolyzate level. At 30% loading levels, overliming was shown to significantly improve ethanol fermentation. It is well known that

treatment of hydrolyzate liquors with strong base can improve fermentation, and it is thought that this is due to elimination of some of the compounds toxic to ethanologenic microorganisms.[12] For the individual toxic compounds studied, the OD values as well as ethanol yields indicated that acetic acid was the most toxic component for *Saccharomyces* at the levels present in the liquid hydrolyzate.[20] Furans become toxic at a greater than 100% levels. Vanillin and syringaldehyde appears to have a slight toxic effect. None of the other phenolic compounds tested were observed to be substantially toxic to *Saccharomyces* at the levels tested. [20]

None of the acetone:water or methanol extracts from solid biomass samples of Red oak, White oak, Yellow-poplar, and treated solids were observed to have a profound toxic effect on *Saccharomyces* ethanol production.[20] Thus, from these experiments it is evident that compared to *Zymomonas mobilis, S. cerevisiae* D5A is very robust to most of the inhibitory compounds present in the mixed hardwood hydrolyzate liquor. Overliming of the hydrolyzate enhanced the glucose fermenting efficiency probably due to removal of some inhibitory compounds, as has been suggested previously.[7] A manuscript describing this work is being prepared for publication as a technical note.

3. Analyses of the NREL Hydrolyzate P960227SD. At this stage, we suggested that a hydrolyzate prepared from less toxic hardwoods such as Yellow-poplar or fast growing wood species such as cottonwood, aspen or poplar would be much more desirable for the bioconversion process. These substrates would avoid problems associated with the tannins and other extractives. However, the problems with acetic acid would still exist as the acetyl content is directly related to the glucuronoxylan content, and the acetyl/glucuronoxylan ratio is invariable for all temperate zone hardwoods. Technical efforts could therefore be aimed at eliminating or minimizing the acetic acid content.

The liquid hydrolyzate (P960227SD) prepared from yellow poplar was analyzed by HPLC utilizing the HPX87-H column.[25] The major compounds which were quantitated were glucose (9.08 ± 0.1 mg/mL), xylose (39.76 ± 1.0 mg/mL), acetic acid (11.52 ± 0.3 mg/mL), HMF (0.76 ± 0.02 mg/mL), and furfural (1.51 ± 0.03 mg/mL).[25] The hydrolyzate was separated using the MTBE extraction method, and various hydrolyzate fractions as well as MTBE extract, and the aqueous phase at different levels was tested for toxic effects toward *Zymomonas mobilis* (Table IV).[26] These results strongly suggested that the freeze dried MTBE extracted material from the acidic hydrolyzate at a 100% level is not extremely toxic towards *Zymomonas* cell growth or ethanol production. The HPLC analysis of the MTBE extract indicated that no acetic acid was present—the reduced toxic effect of the MTBE extract was almost certainly due to the removal of acetic acid from the extracts during the freeze drying process. Residual toxicity would then be due to furans, extractives and lignin degradation products. HPLC analysis indicated the amount of HMF present in a bioassay tube containing 100% level of MTBE extract was 0.19 mg/mL with trace levels of

furfural. This concentration of HMF alone is expected to provide a percent performance in the vicinity of 70-80%.[17]

The ethanol yields indicated that the freeze dried aqueous phase was toxic to *Zymomonas* at a 100% level (Table IV), and the approximate performance of the overlimed hydrolyzate at a 30% level (negative control) matched the 60% aqueous phase loading level. The amount of acetic acid and HMF present in a bioassay tube containing the 60% loading level of aqueous phase was determined to be 1.94 and 0.05 mg/mL, respectively, with only a trace level of furfural detected. This suggests that the toxic effects at a 100% loading may be attributed to the presence of acetic acid. Based on the nonlinear OD data obtained previously, cell growth of *Zymomonas* at this acetic acid level was only about 35% relative to the positive control.[17]

Table IV. *Zymomonas* Growth and Ethanol Yields for Various Hydrolyzate Fractions, MTBE Extracts, and Aqueous Phase after 48 h for the NREL Hydrolyzate P960227SD.

Sample (% Loading Level)	Ethanol (mg/mL)	Percent Performance
Positive Control[a]	17.23-20.72	100
Negative Control (30%)[b]	7.42-7.74	36-45
Ethanol Control	20.78	100
MTBE Extract (MA)[c]		
MA (5%)	17.69	85
MA (15%)	15.69	76
MA (30%)	17.67	85
MA (45%)	16.50	80
MA (80%)	15.56	75
MA (100%)	14.90	72
Aqueous Phase (AA)[d]		
AA (5%)	16.03	93
AA (15%)	12.44	72
AA (30%)	9.38	54
AA (45%)	9.65	56
AA (60%)	7.68	45
AA (100%)	<1	0

[a]Ethanol yields 17.23 and 20.72 mg/mL correspond to the positive control for AA and MA, respectively. [b]Ethanol yields 7.42 and 7.74 mg/mL correspond to the Negative control for MA and AA, respectively. [c]The 100% level of freeze-dried MA represents 3.5 mg/mL. The extracts were dissolved in 0.1 mL of ethanol and introduced to the media (5 mL), thus a ethanol control was included in the setup. [d]100% AA represents 74.6 mg/mL of freeze-dried aqueous phase. The aqueous phase was introduced to the media (5 mL) dissolved in 0.2 mL of water.

Due to the high glucose concentration in the crude hydrolyzate, we felt that some of the performance characteristics of the system may be due to the conversion of glucose to ethanol. Glucose utilization would tend to obfuscate the toxicity results as inhibition of this pathway may not involve the same compounds. Therefore, disappearance of glucose and xylose, and the formation of ethanol in selected bioassay tubes was monitored by HPLC for a 48 h period.[25] A 50% loading level of MTBE and a 30% loading for aqueous extract was chosen for this study, and the results were compared to a positive control. From this experiment complete utilization of glucose after a 24 h period was observed for all the fractions analyzed except for the 30% level of negative control. Thus, the MTBE and aqueous fractions at the levels tested may not substantially affect the glucose utilization of *Zymomonas*.

From the toxicity testing experiments carried out on the aqueous phase and the MTBE extract we believe that minimization of acetic acid and the furans will result in significant increase in the overall xylose to ethanol fermentation, and efforts should be directed toward ameliorating strategies to improve acetic acid removal before the fermentation process.

4. Problems Associated with the Bioassay Experiments. In many instances, the OD values were somewhat susceptible to variation depending on the degree of precipitation in the bioassay tubes. This precipitation was especially problematic at higher levels of hydrolyzate, making the OD values for ascertaining cell growth not very reliable. It is assumed that the precipitation is caused by the formation of phosphate and sulfate salts which have decreased solubility in water. Although the non-linear OD measurements have been corrected with an appropriate blank, when precipitation takes place the method should only be utilized for obtaining a rough estimation of the cell growth of the organism in a specific media. During the execution of toxicity testing of acetone:water and methanol extracts, severe precipitation occurred upon addition of extracts to the media, limiting testing to the 30%—60% levels.

In addition, we were somewhat concerned with the overall reproducibility of the bioassay experiments.[25,26] In some instances, the difference between duplicate experiments were a bit disconcerting, and we believed that part of the problem was due to both the precipitation phenomenon that occurred during the addition of extracts as well as the adhering capabilities of the *Zymomonas* cells. Sedimentation was an unavoidable consequence of some bioassay experiments, and if the cells and precipitate form a mass at the bottom of the tube, poor mixing may lead to less than optimal carbohydrate utilization, affecting the ethanol yields. We addressed this problem previously, and requested some suggestions from NREL to aid in reproducibility. NREL protocols have been modified to occur in larger shake flasks rather than test tube, and the new protocols also utilize pH control. Studues using the modified NREL bioassay are underway and suggest that our lack of high reproducibility may have been caused by both the adhering nature of the cells as well as the decrease in pH during the fermentation process.

5. Analyses of Detoxified Hydrolyzate Samples (NREL Hydrolyzate P960227SD). At this stage of the subcontract, we had quantified most of the major inhibitors and determined many of the factors that influence xylose utilization. The next step was to begin looking at processes that would remove the inhibitors prior to fermentation. Several detoxified samples were prepared by NREL and submitted to our laboratory as unknowns. These were then analyzed by several chromatographic and spectrophotometric techniques. here in our laboratory, and several samples were also submitted to fermentation bioassays at NREL.[27] The results of this effort are summarized in Table V, and the values reported are concentrations or absorbances relative to the original hydrolyzate.

The major inhibitory components in liquid hydrolyzates are carbohydrate decomposition products, lignin degradation products, compounds derived from wood extractives, and metals/minerals in wood.[7,11-14] Thus, the detoxified samples were analyzed by HPLC for carbohydrate and furan determinations, as well as for total phenols, condensed tannins and UV absorbance.[27] According to the fermentability results, a detoxification strategy involving an ion-exchange treatment followed by overliming appears to be the best treatment for removing toxic substances from the hydrolyzate. The fermentability tests also seem to indicate that overliming is necessary for the best fermentation efficiency (although direct evidence is lacking in Table V).

Although overliming is known to remove some inhibitory compounds from hydrolyzate streams the mechanism of action is not clear. It has been suggested that during overliming, inhibitory compounds are precipitated or become bound to the precipitated solid material. Overliming is also reported to reduce levels of furfural, phenolic compounds, and possibly of metal ions.[12,28] The chemical analysis of the hydrolyzates before and after overliming show some differences, although not dramatic. The reason for the increase in acetic acid content after overliming the liquid extracted hydrolyzate (LLE) is not known. The increased values for tannin content upon overliming may due to nonenzymic autooxidative phenolic reactions.[29] Thus, at this point, a firm understanding of the mechanism of overliming cannot be ascertained. In order to see if overliming may influence metal ion concentration in the hydrolyzate, several samples were submitted to metal analyses.

6. Metal Analyses: Overlimed and Crude NREL Hydrolyzate P960227SD. The metal analyses were carried out at the Soil Testing Laboratory at Virginia Tech using Inductively Coupled Plasma Spectrometry (ICP). Analyses were performed for Al, Ca, Fe, Mn, P, Zn, Cu, K, Mg, and Na, and the results are given in Table VI.[30] The data suggest that H_2SO_4 is not a major source for the elements detected in the hydrolyzate. Most of these metals originate from the woody-biomass (see Table 3 in Ref. 30 for inorganic composition of some hardwood species). The iron content in the hydrolyzate is somewhat higher than that found in wood, suggesting that there is some other source for this element, such as reaction vessels. Fermentation

Table V. Analysis of Various Detoxification Strategies for Hardwood Hydrolyzates.

Treatment	Glucose	Xylose	Acetic Acid	HMF	Furfural	Total Phenols	Condensed Tannins	MTBE Extract	A278	Ethanol % theoretical
Untreated Hydrolyzate	100	100	100	100	100	100	100	100	100	---
$Ca(OH)_2$ Neutralized	84.7	86.2	95.7	84.0	98.5	87.5	47.5	114.3	72.4	---
KOH pH Adjusted	84.8	86.3	95.1	93.8	99.2	86.4	119.7	108.1	69.6	---
Standard Overlimed	87.9	87.4	100.9	66.1	69.5	93.1	221.3	87.3	58.5	10.7
High Peroxide and Overlimed	79.8	83.3	105.4	39.1	39.1	38.4	8401.6	34.9	37.8	0.0
High Charcoal	66.7	64.7	53.0	1.4	1.8	1.9	0.0	0.7	1.5	---
High Charcoal and Overlimed	66.2	65.3	54.9	0.0	1.4	2.3	0.0	2.9	1.5	94.1
LLE	92.4	95.3	0.0	20.5	0.0	60.5	82.0	27.0	15.9	---
LLE and Overlimed	95.5	100.4	19.6	3.1	0.0	66.7	113.9	18.2	17.3	18.5
Ion-Exchanged	97.9	92.3	14.6	49.6	50.7	32.4	108.2	19.2	32.3	---
Ion-Exchanged and Overlimed	89.3	91.4	17.5	47.1	41.7	32.1	126.2	34.9	29.4	93.1

Values reported are concentrations or absorbances relative to the untreated hydrolyzate.

studies on wood hydrolyzates using *Candida shehatae* suggest that poor conversion efficiencies can be related to the metal used for processing or indirectly to levels of iron, chromium and nickel.[31] Fermentation studies carried out with *Pachysolen tannophilus* had resulted in poor yields when stainless steel equipment was used.[31]

Table VI. Metal Concentrations (ppm) of Hydrolyzate Samples and References.

Element (Range, ppm)	Hydrolyzate (10-fold dilution)	Overlimed (10-fold dilution)	Hydrolyzate (not diluted)	Overlimed (not diluted)	0.82% H_2SO_4	0.82%H_2SO_4 Overlimed
Aluminum (0.025-5000)	0.7092	0.8983	5.863	6.241	0.0473	0.2128
Calcium (0.100-1500)	35.28	544.40	352.4	4879	0.0444	796.7
Iron (0.005-150)	2.627	1.520	25.57	12.23	0.0039	0.0442
Manganese (0.001-150)	0.8810	0.4310	8.371	3.470	0.0027	0.0217
Phosphorus (0.060-250)	1.916	0.0141	20.15	0.7931	0.0519	0.0158
Zinc (0.004-150)	0.0782	0.0150	0.5690	0.1597	0.0483	-0.013
Copper (0.002-150)	0.0084	0.0056	0.0111	0.0251	0.0000	-0.014
Potassium (0.300-1000)	21.13	21.55	191.9	181.1	0.0860	0.3008
Magnesium (0.010-350)	6.834	10.90	56.74	81.84	0.0065	46.33
Sodium (0.010-200)	3.757	4.037	35.37	34.80	0.0912	1.564

Overliming of the original hydrolyzate removed a substantial amount of iron, manganese, phosphorus and zinc. Increased Mg levels in overlimed hydrolyzates may be due to Mg impurities in the $Ca(OH)_2$ used for overliming. It was interesting to note that a dramatic reduction of phosphorus had occurred during overliming process. However, the most significant difference is the drastic increase in the amount of calcium ions present in the overlimed hydrolyzate. It may be possible that a high concentration of calcium in overlimed hydrolyzates enhances its fermentability, and may also be aided by removal of cations such as iron. It has

been reported that addition of calcium salts to the media enhances the ethanol production of *Zymomonas mobilis* ZM4 at higher concentrations of glucose and sucrose. Addition of calcium carbonate helps in neutralizing the acid produced, therefore acting as a buffer and stabilizing the pH of the media during fermentation.[32] This buffering action enhanced the utilization of glucose and the subsequent production of ethanol indicates calcium may have an indirect effect on ethanol production. Further studies are warrented to more fully understand this phenomenon.

7. *Further Analysis of a Hydrolyzate Prepared from Yellow-poplar.* In addition to the work mentioned above, a detailed analysis was carried out on a Yellow-poplar hydrolyzate (P961014) recently provided from NREL. The data obtained were compared to those of a previously analyzed Yellow poplar hydrolyzate P960227SD (Table VII), as the newer material is more toxic than expected, considering its treatment regime (personal communication, Dr. McMillan). Standard analyses[16] provided concentrations as follows: glucose (6.37 mg/mL ± 0.07), xylose (35.88 mg/mL ± 0.40), acetic acid (8.83 mg/mL ± 0.09), HMF (0.47 mg/mL ± 0.01), furfural (1.17 mg/mL ± 0.02). The glucose, xylose and acetic acid levels were quantitated using RI detection while the furans were analyzed by UV detection. The sugars, acetic acid, and furan levels in this hydrolyzate were somewhat lower than the P960227SD hydrolyzate.[25] The hydrolyzate was also analyzed for total phenols and condensed tannins (Table VII) according to previously described methods.[27] The phenols and tannin contents in the P961014 hydrolyzate were observed to be somewhat similar to those in the P960227SD hydrolyzate.

Table VII. Comparison of the Composition of P961014 and P960227SD Hydrolyzates.

Component (mg/mL)	P960227SD	P961014[b] (% Conc.)
UV (278 nm)[a]	1.62	1.44 (89)
Glucose	9.08	6.37 (70)
Xylose	39.76	35.88 (90)
Acetic acid	11.52	8.83 (77)
Furfural	1.51	1.17 (77)
HMF	0.76	0.47 (62)
Total Phenols[c]	13.41	12.76 (95)
Condensed Tannin[d]	0.12	0.12 (100)
Extractives[e]	3.07	4.32 (141)

[a]UV absorption of samples were determined by diluting 30 µL of hydrolyzate to 10 mL with water. [b]% Conc. values are relative to components in P960227SD. [c]Expressed as syringaldehyde equivalents. [d]Expressed as catechin equivalents. [e]The difference may due to variable losses of volatile components during rotary evaporation.

The MTBE extracted portion of the hydrolyzate P961014 SD was analyzed by reverse phase HPLC. The

chromatogram contained all the peaks that were detected in the MTBE extracted portion of the P960227SD hydrolyzate chromatogram.[25] However, expect for furans, all other peaks were detected at a lesser extent (see chromatograms in Figure 2) in the P961014 hydrolyzate chromatogram. The variation of furan levels in the MTBE extracts are attributed to the variable losses of volatile components during rotary evaporation.

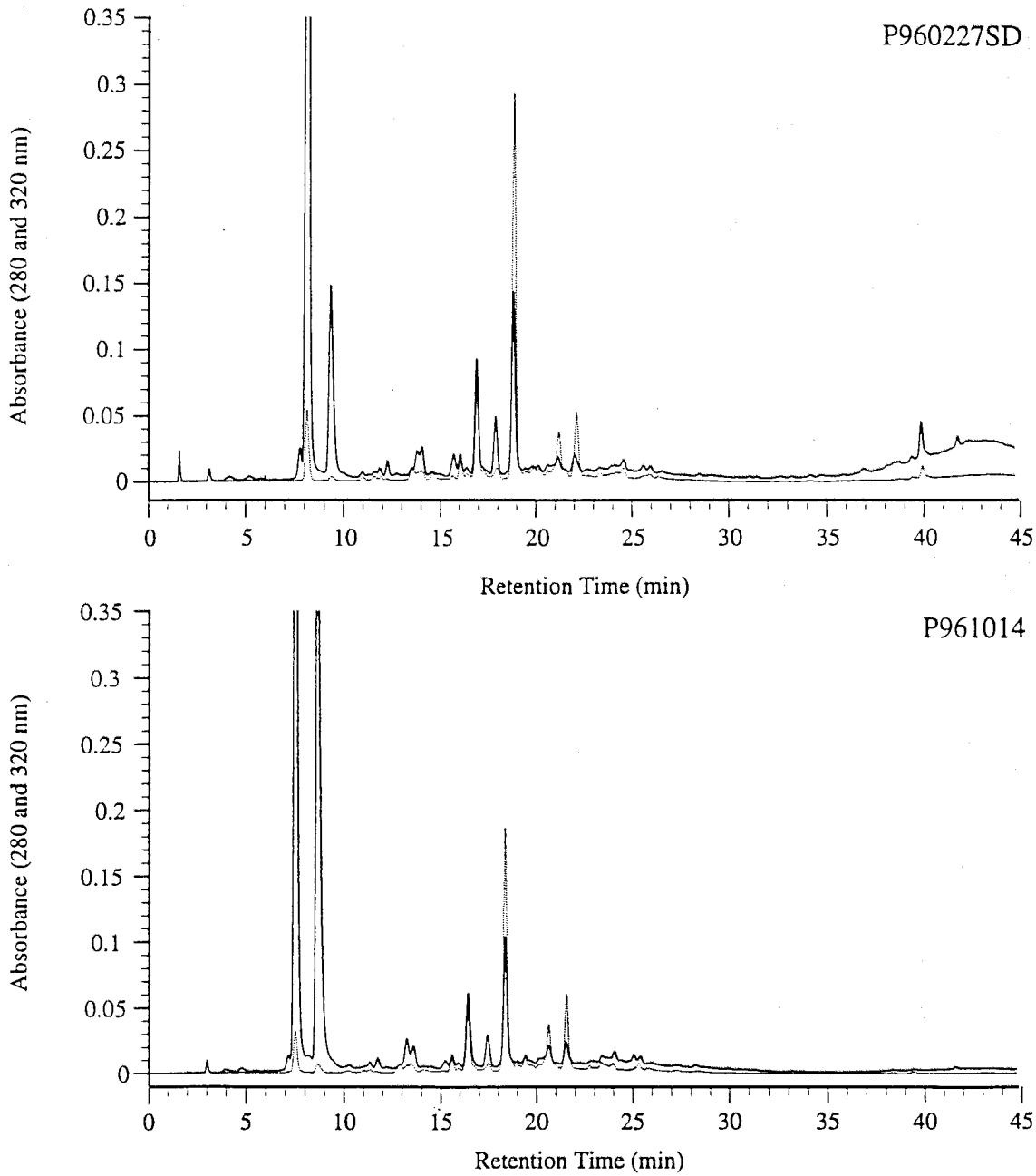

Figure 2. Reverse Phase HPLC Analysis of the MTBE Extracts of Hydrolyzates P960227SD (0.614 mg/mL) and P961014 (0.615 mg/mL) at 280 nm (solid lines) and 320 nm (dotted lines). The two early peaks (7.7 and 8.8 min) are HMF and furfural (respectively).

In addition, a metal analyses were performed on hydrolyzate P961014SD in the "as received" and overlimed forms (Table VIII). Comparison of the data with those obtained for the hydrolyzate P960227SD (Table VI) indicate no significant differences in metal ion concentrations. Overliming appears to remove substantial amount of iron, manganese, phosphorus and zinc from the hydrolyzates. Analyses for heavy metals such as chromium, nickel, and titanium shows only a trace amount to be present, and overliming seems to be helpful in eliminating some nickel from the hydrolyzate. Metals such as lead and arsenic were not detected in the hydrolyzate. What effect these changes have on fermentation efficiency is not known.

Table VIII. Metal Concentrations (ppm) in Hydrolyzate P961014 Samples and References.

Element (Analytical range ppm)	Hydrolyzate	Overlimed	0.82% H_2SO_4	Overlimed 0.82% H_2SO_4
Aluminum (0.025-5000)	7.878	8.616	<0.025	0.2534
Calcium (0.100-1500)	258.2	5677	0.1243	756.5
Iron (0.005-150)	28.96	14.88	0.0495	0.0477
Manganese (0.001-150)	8.504	0.5186	0.0050	0.0397
Phosphorus (0.060-250)	20.81	0.8632	< 0.06	0.0963
Zinc (0.004-150)	1.758	0.1558	0.0204	0.0146
Copper (0.002-150)	0.0896	0.0205	0.0077	0.0102
Potassium (0.300-1000)	197.8	189.3	<0.30	0.3691
Magnesium (0.010-350)	55.49	89.87	<0.01	19.02
Sodium (0.010-200)	20.62	19.76	0.0821	1.347
Chromium (0.007)[a]	0.252	0.270	n.m.[b]	n.m.
Lead (0.04)[a]	n.d.[c]	n.d.	n.m.	n.m.
Nickel (0.014)[a]	0.310	0.164	n.m.	n.m.
Titanium (0.004)[a]	0.023	0.301	n.m.	n.m.
Arsenic (0.05)[a]	n.d.	n.d.	n.m.	n.m.

[a]Minimum Detection Limit, [b]Not Measured, [c]Not Detected.

Currently our efforts are directed toward performing bioassay experiments on the hydrolyzate P961014 using pH adjusted media. The experiments will be performed according to the guidelines provided by NREL scientists, and the results will be provided as an attachment to this report.

MAJOR ACCOMPLISHMENTS

The Tasks listed in the original statement of work for this subcontract are as follows:

Task 1: Generate a list of potential inhibitors.

Task 2: Identify components inhibitory to pentose fermentation organisms.

Task 3: Extend research on Tasks 1 and 2 as necessary.

Task 4: Determine effect of process conditions on major inhibitory components.

Task 5: Recommend and report on amelioration strategies.

The work performed at Virginia Tech for this subcontract has provided some important qualitative and quantitative information on the nature of the hemicellulose hydrolyzate inhibitors. Protocols have been streamlined for both analysis and testing of the fermentation process, and these efforts have led to the testing of several strategies for eliminating a majority of the toxic components. A numerical listing of the major accomplishments of this subcontract are provided below and related to the above Tasks.

1. Protocols for hemicellulose hydrolyzate analysis have been streamlined through a combination of liquid-liquid extraction, HPLC and GC/MS. These efforts included evaluation and improvement of current NREL protocols and the standard protocols developed as part of this subcontract are provided in Appendix A—Experimental Protocols.

2. A ranking of inhibitors has been generated with acetic acid being by far the most toxic component. Oak extracts are also quite toxic and efforts should be made to avoid this substrate. This accomplishment matches with Tasks 1-3.

3. One publication has been accepted for publication in a peer-reviewed journal and another is currently in first draft form. Up to two more publications are anticipated. This accomplishment addresses Tasks 1-3. A manuscript that has been accepted for publication in *Applied Biochemistry and Biotechnology* is included as Appendix B.

4. Testing and analysis of detoxification strategies has been initiated, and standard protocols for analysis have been devised. This accomplishment matches with Tasks 4 and 5.

FUTURE STUDIES AND RECOMMENDATIONS

At present we believe that future studies should be directed toward understanding and mechanisms of detoxification strategies such as overliming, ion exchange, and activated charcoal. These studies may include analyses for trace metals, although the work presented here would seem to indicate that their effect may be minimal. This could easily be tested by performing bioassays at various metal ion concentrations. Ion chromatography may also prove useful in the analysis of neutralized and overlimed hydrolyzates to provide a better understanding of the anions that are present in the system and what potential deleterious roles they may play. It should be pointed out however that we currently do not have the necessary detection equipment at our disposal to rapidly perform these analyses.

The fermentability results have suggested that both ion exchange and charcoal treatment are effective in removal of a majority of the potential inhibitory components, when used in combination with overliming. The major draw back of the charcoal system is that it removed up to 35% of the sugars. Activated charcoal is used in the removal of organic material in wastewater samples, and in the removal of pigments in sugars.

Thus, it may be worthwhile to study the various types of charcoal to optimize the removal of inhibitory components while retaining the sugar fraction. If the charcoal treatment is considered cost effective, attention should be given to optimizing the process. It should be pointed out that previous studies on the development of detoxification strategies with a sugar cane bagasse hemicellulose hydrolyzates indicated that acidified charcoal treatment is effective in removing pigments, with an almost quantitative recovery of sugars.[3]

Whatever detoxification strategy is finally advocated, attention also needs to be given to the robustness of the technique to different substrates. While efforts thus far have dealt with yellow-poplar, this species is only found in eastern portions of the United States. However, if biomass ethanol is to become an economic reality in other regions of the United States, alternative biomass resources would be used. Therefore, studies should be conducted on different substrates such as ryegrass, switchgrass, kenaf and/or a softwood in order to ascertain if the deteoxification strategy developed for yellow-poplar is applicable to all or most available resources.

Listed below are a list of tasks proposed for a one year project aimed at furthering our understanding of ameliorations strategies:

Task 1. A one month project is envisioned to look into a comparison of old and new strains of *Zymomonas*, pH control and its effect on hydrolyzate toxicity, and baffle flask versus test-tube bioassays. Four known inhibitors would be tested: vanillin (100% loading), acetic acid (50% loading), furfural (100% loading) and yellow-poplar acetone/water extract (15% loading). These will be run along with a Positive control and two Negative controls (one with the old *Zymomonas* and one with the new).

Task 2. Detailed investigations (2 months) of the chemistry occuring during or the kinetics of detoxification processing. We envision thoroughly analyzing 3 strategies using the following protocols:

a. Spectrophotometric methods including UV scans (A278), Total Phenol determinations (Folin method) and Condensed Tannin determinations (vanillin/sulfuric acid method).

b. HPX-87H analysis for xylose, glucose, acetic acid, furfural and HMF.

c. Metal analyses as warranted.

d. Some consideration may also need to be given to anion analyses (phosphate, chloride, carbonate, sulfate) and what role these anions play in fermentation performance.

e. Overliming needs to be investigated in more detail. For example, is a five day incubation really required? This is not an industrially-friendly step.

f. Baffle flask bioassays protocols streamlined in Task 1 would be performed in duplicate or triplicate to quantify the strategies investigated.

Task 3. Studies to assess efficacy of detoxification processes on different substrates. A goal of Task 2 is to streamline analysis and bioassay protocols to handle any biomass pretreatment hydrolyzate. Therefore analysis of any sample should be possible. Compositional analyses including the spectrophotometric methods in combination with the HPX-87H sugar/organic acid/furan determinations would be conducted. One month per substrate is envisioned.

Task 4. Development of improved techniques for inhibitor identification. It is hoped the the protocols outlined in Task 2 in combination with the results obtained in Task 3 would be sufficient to allow us to PREDICT the toxicity of biomass prehydrolyzates. While we cannot say at this time if the protocols we use at present will be sufficient to achieve such a level of understanding, time should be reserved at the end of the project (1-2 months) for such an endeavor.

REFERENCES

1. South, C. R.; Hogsett, D. A.; and Lynd, L. R., Continuous Fermentation of Cellulosic Biomass to Ethanol, *Appl. Biochem. Biotechnol*, **39/40** (1993), 587-600.

2. Beavan, M.; Zawadzki, B.; Droniuk, R.; Lawford, H.; Fein, J. Comparative Performance Trials with Yeast and *Zymomonas* for Fuel Alcohol Production from Corn *Appl. Biochem. Biotechnol.*, **20/21** (1989), 437-448.

3. Gong, C. S.; Chen, C. S.; Chen, L. F.; Pretreatment of Sugar Cane Bagasse Hemicellulose Hydrolyzate for Ethanol by Yeast *Appl. Biochem. Biotechnol.* **39/40**, (1993), 83-88.

4. Jeppsson, H.; Yu, S.; Hahn-Hagerdal, B. Xylulose and Glucose Fermentation by *Saccharomyces cerevisiae* in Chemostat Culture *Appl. Envi. Microbio.*, **62** (1996), 1705-1709.

5. Olsson, L.; Hahn-Hagerdal, B. Fermentation Performance of Bacteria and Yeasts in Lignocellulose Hydrolyzates *Process Biochem.* **28** (1993), 249-257.

6. Hahn-Hagerdal, B.; Linden, T,; Senac, T.; Skoog, K. Ethanolic Fermentation of Pentoses in Lignocellulose Hydrolyzates *Appl. Biochem. Biotechnol.* **28/29** (1991), 131-144.

7.McMillan, J. D. Conversion of Hemicellulose Hydrolyzates to Ethanol, In Bioconversion for Fuels, (M. E. Himmel, J. O. Baker and R. P. Ovrend, Eds). *ACS Symp. Ser.* **556** (1994), Washington DC, pp.411-437.

8. Prieto, S,; Clausen, E. C.; Gaddy, J. L. Improved hydrolysis Process from the Saccharification of Biomass *Proceedings of the 8th Symposium on Biotechnology for fuels and Chemicals*, 1986, 123-133.

9. Petterson, R. C. The Chemical Composition of Wood, In The Chemistry of Solid Wood, (R. M. Rowell, Ed.) *ACS Symp. Ser.* 207, Washington, DC, pp 57-126.

10. Zhang, M.; Eddy, C.; Deanada, K.; Finkelstein, M.; Picataggio, S. Metabolic Engineering of a Pentose Metabolism Pathway in Ethanologenic *Zymomonas mobilis Science*, **267** (1995), 240-243.

11. Tran, A. V.; Chambers, R. P. Ethanol Fermentation of Red Oak Prehydrolyzate by the Yeast *Pichia stipitis* CBS 5776. *Enzyme Microb. Technol.*, **8** (1986), 439-444.

12. Frazer, F. R.; McCaskey, T. A. Wood Hydrolyzate Treatments for Improved Fermentation of Wood Sugars to 2,3-Butanediol, *Biomass*, **18** (1986), 439-444.

13. Lenored, R. H.; Hajny, J. G. Fermentation of Wood Sugars to Ethyl Alcohol *Ind. Eng. Chem.*, **37** (1945), 390-395.

14. McCaskey, T. A.; Rice, M. D.; Smith, R. C. Improved Fermentation of Wood Hydrolyzate to Ethanol by Removal of Compounds Inhibitory to *Paschysolen tannophius.* In Biomass Energy Development, (W. H. Smith, Ed,), Plenum, New York, 1986, 573-585.

15. Antal, M. J.; Leesomboon, T.; Mok, W. S.; Richard, G. N. (1991), *Carbohydr. Res.*, **217** (1991), 71-85.

16. Helm, R. F.; Ranatunga, T. D. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 01 March 1995 to 30 April 1995.

17. Ranatunga, T. D.; Jervis, J.; Helm, R. F. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 31 October 1994 to 30 October 1995.

18. Ranatunga, T. D.; Jervis, J.; Helm, R. F. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 01 May 1995 to 30 June 1995.

19. Ranatunga, T. D.; Jervis, J.; Helm, R. F. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 01 July 1995 to 30 August 1995.

20. Ranatunga, T. D.; Jervis, J.; Helm, R. F. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 01 January 1996 to 29 February 1996.

21. NREL Chemical Analysis and Testing Procedures: Experimental Protocols for Assessing the Toxicity of Hemicellulose Hydrolyzates using *Zymomonas mobilis CP4(PZB5)* and *Saccharomyces cerevisiae* D5A.

22. Ranatunga, T. D.; Jervis, J.; Helm, R. F. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 01 November 1995 to 31

December 1995.

23. Rowe, J. W.; Conner, A. H. Extractives in Eastern Hardwood-A Review, Gen. Tech. rep. FPL-18, USDA Forest Products Laboratory, 1979.

24. Scalbert, A. In Plant Polyphenols, Hemingway, R. W. Laks, P. E.; ed.; Plenum Press, New York, 259-280.

25. Ranatunga, T. D.; Jervis, J.; Helm, R. F. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 01 March 1996 to 30 April 1996.

26. Ranatunga, T. D.; Jervis, J.; Helm, R. F. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 01 May 1996 to 30 June 1996.

27. Ranatunga, T. D.; Jervis, J.; Helm, R. F. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 01 July 1996 to 31 August 1996.

28. Strickland, R. C.; Beck, M. J.; Effective Pretreatment Alternatives for the Production of Ethanol from Hemicellulose Hardwood Hydrolyzates, *Appl. Biochem. Biotech.*, 17/18 (1988), 357-369.

29. See for example: Tulyathan, V.; Boulton, R. B.; Singleton, V. L. Oxygen Uptake by Gallic Acid as a Model for Similar Reactions in Wines, *J. Agric. Food. Chem.*, **37** (1989), 844-849; Cilliers, J. J. L.; Singleton, V. L. Nonenzymic Autooxidative Phenolic Browning Reactions in Caffeic Acid Model System, *J. Agric. Food Chem.*, **37** (1989). 890-896.

30. Ranatunga, T. D.; Jervis, J.; Helm, R. F. Identification of Inhibitory Components in Dilute Acid Pretretated Lignocellulosic Materials, Bimonthly Technical Status Report, Reporting Period 01 September 1996 to 31 October 1996.

31. Beck, M. J. Factors Affecting Efficiency of Biomass Fermentation to Ethanol, *8th Symposium on Biotechnology for Fuels and Chemicals*, pp 617.

32. Sreekumar, O.; Basappa, S. C. Effect of Calcium and Sodium Salts on Ethanol Production in High Sugar Fermentation by Free Cells of *Zymomonas mobilis. Biotechnology Lett.*, **14** (1992) 511-514.

Appendix A—Experimental Protocols

Protocol VT-1 *Extraction and Analysis of the Liquid Hydrolyzate Organic Fraction .*

a. Solvent Extraction Method for Preparing an Organic Fraction from the Liquid Hydrolyzate.
b. Preparation of Extracts for Toxicity Testing.
c. Reverse Phase HPLC analysis of the Extracts.
d. GC/MS Analysis of the Extracts.

Protocol VT-2 *Determination of Total Phenols in Hydrolyzate Samples.*

a. Preparation of a Calibration Curve with Gallic Acid and/or Syringaldehyde.
b. Total Phenol Determination.

Protocol VT-3 *Determination of "Condensed Tannins" in Hydrolyzate Samples.*

a. Preparation of a Calibration Curve with (+)-catechin hydrate.
b. Tannin Determination.

VT-1. Extraction and Analysis of the Liquid Hydrolyzate Organic Fraction .

The procedure was adopted from the method described by Örsa and Holmbom (Örsa. F.; Holmbom, B. A Convenient Method for the Determination of Wood Extractives in Papermaking Process Waters and Effluents. *J. Pulp and Paper Sci.*, **20** (1994), 361-366). The method has been used in the analysis of thermomechanical pulp (TMP) and paper mill wastewaters.

1a. Solvent Extraction Method for Preparing an Organic Fraction from the Liquid Hydrolyzate.

1. Extract the acid hydrolyzate (20 mL) three times with methyl *t*-butyl ether (MTBE, HPLC-grade, 50 mL).

2. Combine the organic layers, and add a small amount of granular anhydrous sodium sulfate to remove any dissolved water. Filter, and finally evaporate the MTBE under reduced pressure using a rotary evaporator (temperature of bath: approximately 40 °C)

3. Save the aqueous phase for toxicity studies.

4. Express the MTBE extract yield in mg/mL. Note: The yield depends on the evaporation time; rotary evaporation results in the loss of volatile materials such as acetic acid and furans.

5. An organic fraction can also be prepared from a pH adjusted hydrolyzate (pH 6.5) fraction using the above method. The pH can be adjusted using a 2M KOH solution.

1b. Preparation of Extracts for Toxicity Testing.

1. MTBE Extracts

i. Freeze dry the MTBE extract for at least 48 h to remove any trace volatiles remaining in the sample. Obtain yield.

ii. Dissolve a known amount of freeze-dried MTBE extract in a known volume of distilled ethanol, and use in bioassay experiments.

2. Aqueous Phases

i. Rotary evaporate the acidic/neutralized aqueous phase recovered from the MTBE extraction to remove any MTBE dissolved in the sample.

ii. Neutralized the aqueous phase to pH 6.5 using 2M KOH.

3. Freeze-dry the samples; yields can be expressed as mg/mL. If necessary, freeze dried samples can be desiccated and stored in a freezer.

4. Dissolve a known amount of freeze dried sample in a known amount of DI water, and use for bioassay experiments.

1c. Reverse Phase HPLC Analysis of the MTBE Extracts.

While the composition of the MTBE and aqueous samples can be determined by HPLC using a HPX87-H column (NREL Chemical Analysis and Testing Procedures: HPLC Analysis of Liquid Fractions of Process Samples for Organic Acids, Glycerol, HMF, and Furfural—LAP015), reverse-phase HPLC is quite useful for analysis of the organic fraction. This analysis is based on previous work: Kermasha, S.; Goetghebeur, M.; Dumont, J. Determination of Phenolic Compounds Profiles in Maple Products by High-Performance Liquid Chromatography, *J. Agric. Food Chem.* , **43** (1995), 708-716.

1. Prepare samples for HPLC analysis by dissolving MTBE extract (about 30-40 mg) in methanol (1 mL or less). Adjust the final volume of the sample to 50 or 100 mL using DI water (Preparing samples in pure methanol causes peak tailing).

2. Analyze the samples by reverse phase HPLC using the following conditions:

Column: C_{18} column (Whatman RAC-II, 5-mm particle size)

Flow rate: 0.75 mL/min

Solvent A: HPLC grade Methanol

Solvent B: 0.2% (v/v) Trifluoro acetic acid (TFA)

Detection: 280 and 320 nm.

Gradient: The linear gradient consists of an initial concentration of 100% solvent B. Begin the linear gradient at 1 min so that at 40 min the concentration of solvent B decreases to 5%. Hold at this concentration for 5 min (40-45 min), and bring the gradient back to the original concentration in 5 min. After a 10-min reequilibriation, the system is ready for another injection.

1d. GC/MS Analysis of the Extracts.

The phenolics and furans present in the samples can also be converted to volatile trimethylsilyl derivatives for GC/MS analysis (Örsa. F.; Holmbom, B. A Convenient Method for the Determination of Wood Extractives in Papermaking Process Waters and Effluents. *J. Pulp and Paper Sci.*, **20**(1994), 361-366).

1. React the MTBE extract (about 2-3 mg) with 150-200 µL of bis-(trimethylsilyl)trifluoroacetamide (BSTFA)/trimethylcholorosilane (TMCS) (99:1) reagent (Supleco Inc.) at 45-50 °C for 20-30 min.

2. Diluted the reaction product with MTBE (if necessary), and analyze the samples by GC/MS using the following conditions:

Column: DB-5 capillary column (J & W Scientific) with a 0.25 µm film thickness.
Carrier gas: He; Head Pressure - 10 psi
Column Temperature: Initial - 50 °C; Final - 325 °C at 10 °C/min.
Injector Temperature: 200 °C

VT-2 Determination of Total Phenols in Hydrolyzate Samples.

Total phenols in hydrolyzate samples can be estimated using the Folin-Ciocalteu spectrophotometric method (Scalbert, A.; Monties, B.; Janin, G. Tannin in Wood: Comparison of Different Estimation Methods, *J. Agric. Food. Chem.*, **37** (1989), 1324-1329. Swain, T.; Hillis, W. E. The Quantitative Analysis of Phenolic Constituents, *J. Sci. Food Agric.*, 10 (1959), 63-68). The method is based on formation of a colored complex with metal ions, caused by the reduction of a phosphotungstic-phosphomolybdic reagent (Folin-Ciocalteu Reagent). The total phenols in the samples are expressed relative to a standard, in this case syringaldehyde or gallic acid. The value determined is termed equivalents/mL of hydrolyzate. Either gallic acid or syringaldehyde can be chosen as a standard depending on which compound is actually present in the hydrolyzate. In some instances, high amounts of sugars may interfere with the assay. To verify this, the assay needs to be performed on a synthetic hydrolyzate. The synthetic hydrolyzate should represent the actual amounts of sugars, acetic acid, and furans in the original hydrolyzate sample.

2a. Preparation of the Calibration Curve.

1. Adjust the wavelength of the spectrophotometer to 760 nm.
2. Prepare the Folin Ciocalteu Reagent by diluting 10-fold with DI water. The reagent is commercially available from Sigma Chemical Co.
3. Prepare a sodium carbonate solution (75g/L).
4. Prepare a series of standard solutions (10-60 µg/mL) using a stock solution of either gallic acid or syringaldehyde (Prepare the stock solution by dissolving a known amount of compound in a minimum amount of methanol, and diluting to a known volume with DI water).
5. Add diluted Folin Ciocalteu reagent (2.5 mL) to each standard solution (0.5 mL) in screw capped test tubes, and mix well.
6. Prepare a reference (blank) by adding diluted Folin Ciocalteu reagent (2.5 mL) to DI water (0.5 mL) in a screw capped test tube.

7. After 5 min, add 2 mL of sodium carbonate solution (75g/L) to each tube (reference and standards).

8. Place the tubes (reference and standards) in a 50 °C water bath for 5 min.

9. After 5 min transfer the tubes to a cold water bath.

10. Zero the spectrophotometer using the reference. Record the absorbance of each standard solution at 760 nm.

11. Prepare a standard curve by plotting the absorbance vs concentration of standard solutions (µg/mL).

2b. Total Phenol Determination.

1. Adjust the wavelength of the spectrophotometer to 760 nm.

2. Dilute the original hydrolyzate samples with DI water to a known volume.

3. Add 2.5 mL of the diluted Folin-Ciocalteu reagent to the dilute sample (0.5 mL) in a screw capped test tube.

4. Prepare a reference (blank) according to Step 6 in Section 2a.

5. After 5 min, add 2 mL of sodium carbonate solution (75g/L) to the tubes (reference and sample).

6. Place the tubes (reference and sample) in a 50 °C water bath fro 5 min, and then transfer to a cold water bath.

7. Zero the spectrophotometer with the reference, and record the absorbance of samples at 760 nm.

8. The absorbance of the samples should be in the linear range of the calibration curve (absorbance below 0.5). If not dilute the original hydrolyzate samples to a suitable concentration, and repeat the assay.

9. Using the standard curve and the dilution factor, calculate the amount of total phenols in the sample. Values are expressed as mg/mL gallic acid or syringaldehyde equivalents.

VT-3 Determination of "Condensed Tannins" in Hydrolyzate Samples.

Condensed tannins in the hydrolyzate can be estimated using the Vanillin/H_2SO_4 method described by Swain and Hillis (Scalbert, A.; Monties, B.; Janin, G. Tannin in Wood: Comparison of Different Estimation Methods, *J. Agric. Food. Chem.*, **37** (1989), 1324-1329. Swain, T.; Hillis, W. E. The Quantitative Analysis of Phenolic Constituents, *J. Sci. Food Agric.*, 1**0** (1959), 63-68.). The assay is useful in determining low amounts of proanthocyanidins in samples. In this method vanillin forms a complex with proanthocyanidins that absorbs at 500 nm. The major functional group required is *meta*-disposed hydroxyl groups on an aromatic ring. The H_2SO_4 acts as a catalyst in the reaction. The condensed tannins present in the samples are expressed in catechin equivalents/mL of sample. Thus, as with the previous protocol (VT-2) this method can only be utilized to estimate relative tannin contents in samples. The possible interferences for this test by sugars, acetic acid, and furans in the hydrolyzate can be tested by carrying out the assay with a synthetic hydrolyzate. The synthetic hydrolyzate should contain the same amounts of sugars, acetic acid, and furans detected in the original hydrolyzate.

3a. Preparation of Standard Curve.

1. Adjust the wave length of the spectrophotometer to 500 nm.
2. Prepare a vanillin/H_2SO_4 reagent: vanillin (1g) in 70% H_2SO_4 (100 mL). Cool the reagent on ice. The reagent needs to be freshly prepared.
3. Prepare series of catechin standards (5-25 µg/mL) using a stock solution of (+)-catechin hydrate (Aldrich or Sigma). Note: The degree of hydration is somewhat variable from lot to lot of catechin. There is no simple way to dry catechin without potentially modifying the structure. Therefore the calibration curve will be somewhat dependent on the catechin source.
4. Pipette 1 mL of each standard to a series of test tubes, and cool them on ice.
5. Prepare a reference (blank) using 1 mL of DI water, and cool on ice.
6. Add vanillin/H_2SO_4 reagent (2 mL) to the tubes (standards and reference).
7. Immediately transfer all assay tubes (standards and reference) to a 20 °C water bath, and allow to react for 15 min.
8. After 15 min, transfer the assay tubes to an ice bath.
9. Zero the spectrophotometer using the reference (blank). Record the absorbance of standard solutions at 500 nm.
10. Construct the calibration curve by plotting the absorbance vs catechin (mg/mL)

3b. Estimation of Condensed Tannins in Hydrolyzate Samples.

1. Adjust the wave length of the spectrophotometer to 500 nm.
2. Dilute the hydrolyzate samples with DI water to a known volume.
3. Place the dilute sample solution (1mL) in a test tube, cool on ice.
4. Prepare a reference (blank) using DI water (1mL), cool on ice.
5. Add 2 mL of vanillin/H_2SO_4 reagent to each tube (sample and reference), and mix well. Immediately transfer to a 20 °C water bath, and allow to react for 15 min.
6. Zero the spectrophotometer with the reference, and record the absorbance of sample at 500 nm.
7. The absorbance of the sample should be in the linear range of the calibration curve. If not dilute the original hydrolyzate samples to an appropriate concentration, and repeat the assay.
8. Calculate the amount of catechin in the sample using the calibration curve and the dilution factor. Values are expressed in catechin equivalents/mL of sample.

APPENDIX B—Revised and Accepted Manuscript Submitted to *Applied Biochemistry and Biotechnology*

Identification of Inhibitory Components Toxic Toward *Zymomonas mobilis* CP4(pZB5) Xylose Fermentation

Thilini D. Ranatunga, Judith Jervis and Richard F. Helm*
Fralin Biotechnology Center, Department of Wood Science and Forest Products, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0346

James D. McMillan and Christos Hatzis
Biotechnology Center for Fuels and Chemicals, National Renewable Energy Laboratory, Golden, CO 80401-3393.

ABSTRACT

Zymomonas mobilis CP4(pZB5) is a recombinant bacterium that can produce ethanol from both xylose and glucose. The ethanol-producing efficiency of this organism is substantially impeded by toxic substances present in pretreated hydrolyzates or solid biomass substrates. Acetic acid and furfural (a pentose degradation product) are highly toxic to this organism at levels envisioned for a pretreated hardwood liquid hydrolyzate. In addition, lignin degradation products and 5-hydroxymethylfurfural (a hexose degradation product) have a moderately toxic effect on the organism. Of the compounds studied, organic acids and aldehydes were found to be more inhibitory than lignin acids or the one alkaloid studied. Acetone:water and methanol extracts of solid biomass samples from red oak, white oak, and yellow poplar are toxic to *Zymomonas* cell growth and ethanol production, with the extracts from white oak being the most toxic.

Index Entries: *Zymomonas,* recombinant, ethanol, oak, toxicity, yellow-poplar, xylose

* *Author to whom all correspondence should be addressed. Phone: 540-231-4088, FAX: 540-231-7126, E-mail: helmrf@vt.edu.*

INTRODUCTION

Zymomonas mobilis is an ethanologenic bacterium, generally considered an economical high-performance biocatalyst for ethanol production from glucose originating from various substrates (*1-4*). The CP4 (pZB5) mutant of this organism has been genetically modified to also produce ethanol from the pentose sugar, xylose (*5*).

Lignocellulosic materials from woody biomass have been targeted as a potential substrate for industrial ethanol production. Ethanologenic microorganisms such as *Z. mobilis* and *Saccharomyces cerevisiae* have been favorably used to convert biomass to ethanol (*6-9*). However, when considering the economics of ethanol production, these microorganisms must be able to completly convert all potential carbohydrates in lignocellulosics. Xylose is a major hydrolysis product of hardwood hemicelluloses, and constitutes 10 to 26% of the dry weight of temperate zone hardwoods (*10*). This pentose is a difficult sugar for most microorganisms to convert to ethanol (*11,12*). The combined xylose- and glucose- fermenting capabilities of the recombinant *Z. mobilis* CP4 (pZB5) make this organism a potentially ideal biocatalyst for economical ethanol production.

The conversion of woody biomass to ethanol typically involves a two-stage process. An initial dilute sulfuric acid treatment of wood affords deacetylation and depolymerization of the hemicelluloses. The resulting sugar solution is fermented to ethanol, and the remaining solid material is submitted to a simultaneous saccharification and fermentation process to convert the available glucose.

The ethanol fermentation efficiency of *Z. mobilis* CP4 (pZB5) with the hemicellulose hydrolyzate is substantially hindered by the toxic substances present in this stream. In addition to acetic acid, many other inhibitory compounds such as organic acids, phenolics, and carbohydrate degradation products are suspected to be present in the hydrolyzates at levels that will affect the overall xylose-to-ethanol conversion process (*13*). Xylose fermentation inhibition seriously jeopardizes the economic success of wood-to-ethanol producing facilities.

Efforts are now underway to improve process yields by increasing the efficiency of carbohydrate conversion to ethanol. There are several reasons for inefficient conversion, and the quantitative aspects of these limitations are not completely understood. The objective of this study was to obtain a firmer, quantitative understanding of the role inhibitors play in the conversion of xylose to ethanol by the recombinant *Zymomonas CP4* (pZB5), and the results of this investigation are presented below.

EXPERIMENTAL METHODS

General

The recombinant *Z. mobilis* CP4 (pZB5) used in this study was obtained from M. Zhang at the National Renewable Energy Laboratory (NREL). The bacterium was stored at -70^{0}C in cryovials containing RM media (1X conc., yeast extract 10 g/L, KH_2PO_4 2g/L) and glycerol (20% W/V). Inoculum preparation was as follows. One thawed vial of *Z. mobilis* was mixed with a medium that contained RM medium (10X conc., 20 mL), D-glucose (500 g/L, 10 mL), D-xylose (500 g/L, 10 mL), and tetracycline stock solution (12 mg/mL, 0.25 mL). The total liquid volume was adjusted to 200 mL with deionized (DI) water. The cells were cultivated in a shaker at 30^{0}C (18 h), spun down for 10 min at 4000 rpm, and the supernatant was discarded. The cells were resuspended in sterile RM media (10X conc., 5 mL) plus DI water (45 mL), and subsequently diluted with DI water to to a final optical density ($OD_{600\ nm}$) value of 25-30.

Toxicity testing was carried out in sterile 12 mL screw-capped test tubes containing 10 mL of "positive" control medium (yeast extract, 10g/L; KH_2PO_4, 2g/L; xylose, 44.7g/L; glucose, 2.4g/L; tetracycline, 1.2 mg/mL). The initial sugars in the positive control represent the amounts in the liquid hydrolyzate (preparation of which is described in the next section). The inhibitory compounds were added to the culture tubes dissolved either in ethanol (0.1 mL) or in ethanol:water (1:1 mixture). Each tube was inoculated with *Zymomonas* to achieve an initial net OD_{600nm} of 0.2. Finally, the tubes were incubated at 30^{0}C in a shaker with agitation for up to 72 h. The cell growth was monitored by measuring the nonlinear OD of the culture tubes at 600 nm using a Milton-Roy

Spectronic 601 spectrophotometer (slit width ~ 5 mm). The nonlinear OD measurements for each test condition were corrected with an appropriate blank control (medium without *Zymomonas* cells). The net OD values reported are obtained by subtracting the OD of test sample from the OD of medium blank. Toxic effects were also monitored by determining ethanol concentrations with a Varian gas chromatograph equipped with a flame ionization detector, and a Porapak Q 80/100 glass column (2 m x 4 mm i.d.) packed in-house. Isopropanol was used as the internal standard.

Preparation of the Liquid Hydrolyzate

The mixed sawdust feedstock (1:1:1 red oak: white oak: yellow poplar) was milled and pre-screened to pass through an 8-mm screen. The pretreatment run was conducted at a solids concentration of 20% (based on dry weight) using 0.82% (w/w) sulfuric acid at 160°C for 10 min. A high-solids paddle reactor, which is a steam-jacketed, horizontal cylindrical vessel of approximately 100 L total volume fitted with and internal paddle mixer, was utilized in this run. Biomass and deionized water are charged to the vessel through a flanged access port. After the vessel is sealed, steam is supplied to the shell to heat the vessel contents. When the target temperature is approached, a calculated amount of sulfuric acid solution is pumped into the reactor and the reactor is held at the desired temperature for the designated time. Then, the reactor is cooled down by water supplied through the shell and its contents are discharged and separated by filtration. The liquid hydrolyzate and the pretreated solids were stored refrigerated at 4°C.

Preparation of Various Hydrolyzate Samples for Toxicity Testing

The overlimed hydrolyzate was prepared by adjusting the hydrolyzate pretreatment liquor to pH 10.0-10.5 with solid $Ca(OH)_2$. The hydrolyzate was then kept at 50°C for 30 min. Finally, the pH was adjusted to 7.0 using 96% (v/v) H_2SO_4. The mixture was sterile filtered through a 0.2-μm sterile filter and stored at 4°C for a minimum of 5 days. The direct neutralized hydrolyzate was prepared by neutralizing with $Ca(OH)_2$ to achieve a final pH of 6.0. On the day of the experiment the hydrolyzates, overlimed or direct neutralized (30 mL), were combined with yeast extract (1g),

KH_2PO_4 (200 mg), tetracycline (0.1 mL stock), xylose (3.1 g), glucose (168 mg) and water to achieve a sugar concentration the same as the liquid hydrolyzate from the reactor.

An organic phase was also prepared from the liquid hydrolyzate by extracting with methyl *tert*-butyl ether (MTBE) (*14*). Essentially the crude hydrolyzate (15 mL) was extracted with MTBE (50 mL) three times, and the combined organic phase was evaporated under reduced pressure and subsequently under high vacuum.

Processing of Untreated and Treated Solid Biomass Substrates

The freeze-dried, untreated (red oak, white oak and yellow poplar) and treated (mixed hardwood) solid biomass samples were Wiley-milled to a fine powder (1-mm screen), with precautions to minimize the sample heating. The resulting powders were subjected to three 48-h, room temperature acetone:water (7:3) extractions. The combined extracts (obtained by filtration) were evaporated under reduced pressure at room temperature to remove acetone, with the water subsequently removed by freeze drying. After the extractions, the fibers were air dried, weighed and subjected to 48-h soxhlet extractions with methanol. The methanol extracts were evaporated under reduced pressure followed by freeze-drying. The treated mixed hardwood was processed in the same fashion except that prior to processing the material was washed with distilled water until the wash water and distilled water pHs were the same.

Chemical Analyses

Glucose, xylose, acetic acid, ethanol, HMF, and furfural concentrations present in the hydrolyzate were determined using a Waters HPLC system equipped with a refractive index (RI) detector, and an HPX87-H analytical column (Bio-Rad). The column was kept at 65 °C, and 0.01N H_2SO_4 was used as the mobile phase with a flow rate of 0.6 mL/min. All compounds were quantitated using an external standard method.

The gradient HPLC analyses were carried out using a Gilson HPLC system, equipped with a dual wavelength UV-detector (Model 119) and a C_{18} column (Whatman RAC-II, 5-mm particle

size). The gradient used is based on a previously reported method (*15*) which utilizes methanol (solvent A) and 0.2% trifluoroacetic acid in water (solvent B). The linear gradient consisted of an initial concentration of 100% solvent B. The linear gradient was begun at 1 min so that at 40 min the concentration of solvent B had decreased to 5%. This concentration was held for 5 min (40-45 min) and the gradient was returned to the original concentration in 5 min. After a 10-min re-equilibration, the system was ready for another injection. The flow rate was maintained at 0.75 mL/min, and the dual wavelength UV detector set at 280/320 nm, with the 280-nm data channel used for data collection.

The GC/MS analysis of derivatized extracts was carried out using a Fisions gas chromatograph/mass spectrometer equipped with a DB-5 capillary column (J & W Scientific). The extracts were silylated using a commercially available BSTFA/TMCS (99:1) reagent (Supleco Inc.) as described previously (*14*).

The carbohydrate analyses of fiber samples were carried out by a method adopted from Kaar and co-workers (*16*). The Klason and acid-soluble lignins as well as ash determinations were performed according to standard protocols (*17,18*). All the samples analyzed for carbohydrates were quantitated using a Gilson HPLC system equipped with an Aminex HPX-87P column (Bio-Rad, maintained at 85°C) and an RI detector. HPLC grade water was used as the eluent at a flow rate of 0.6 mL/min, and the samples were injected to the column through a 20-μL loop. Sugars were quantitated using an external standard analysis.

RESULTS AND DISCUSSION

The major compounds present in the pretreated liquid hydrolyzate prepared from the mixture of red Oak, white Oak, and yellow-poplar were glucose (2.40 ± 0.48 mg/mL), xylose (44.75 ± 2.02 mg/mL), acetic acid (9.22 ± 0.43 mg/mL), and furfural (0.91 ± 0.051mg/mL). The amount of 5-hydroxymethylfurfural (HMF) in the hydrolyzate was negligible compared to the other three components. Acetic acid, furfural, and HMF are formed during the pretreatment process. The xylan component of wood is deacetylated and depolymerized, and under well-

controlled conditions provides xylose, acetic acid, and 4-*O*-methyl-D-glucuronic acid. However, such conditions are not achieved during industrial processing and some of the xylose is degraded to furfural (*19*). Furthermore, glucuronoxylans of hardwoods contain an average of 1 uronic acid group per 7 xylose residues (*20*). The 4-*O*-methyl-α-D-glucopyranosiduronic acid linkage to xylose is the most acid-stable of all glycosidic linkages in wood (*21*). Thus, approximately 1/7 of all xylose in temperate zone hardwoods can potentially remain unavailable for fermentation. HMF results from the dehydration of a portion of the hexoses formed during the pretreatment process.

Purification and Analysis of Solid Biomass Samples

The predominant extractives in the oaks are hydrolyzable tannins, and the compounds in yellow-poplar range from alkaloids (glaucine) to sesquiterpenes and lignans (*22*). As these compounds provide passive protection for pathogen invasion in trees (*23*), it can be expected that some of the extractives may also be toxic to *Zymomonas* and may be found in the hemicellulose hydrolyzate stream. Biomass samples were first extracted with acetone:water (7:3) to remove most of the hydrolyzable tannins (*24*), and subsequently by a hot methanol soxhlet extraction. The yields for the acetone:water and methanol extractives are given in Table 1. Interestingly, the treated solid sample afforded the highest percentage of acetone:water extracts, which is probably due to lignin degradation products that are insoluble in acidic water but soluble in acetone:water.

Separation and Analysis of the Pretreatment Liquid Hydrolyzate

Pretreatment hydrolyzate liquors contain variety of inhibitory components that are toxic to various microorganisms (*11*): metals/minerals, carbohydrate decomposition products, lignin degradation products and/or compounds derived from wood extractives are all potential inhibitors (*25-27*). In order to separate the non-polar organics from the polar, fermentable fraction, the hydrolyzate was fractionated with methyl *tert*-butyl ether (MTBE) (*14*).

The amount of material recovered from the extraction was reproducible (3.14 ± 0.08 mg/mL), and HPLC analysis of the aqueous phase and the MTBE extract confirmed that MTBE

separated most of the phenolics and furans from the polar carbohydrates (which remain in the aqueous fraction).

The GC/MS analysis of the trimethylsilylated MTBE-extracts revealed gallic acid, HMF, vanillin, protocatechuic acid and syringaldehyde. These compounds were identified by comparing the mass spectral fragmentation patterns and retention times with those of commercially available compounds. Reverse-phase HPLC analysis of the MTBE extracted material confirmed the GC/MS study, and it was further determined that gallic acid, HMF, and furfural were the major compounds present (Figure 1). These peaks were identified by comparing the retention times with authentic compounds, and by spiking the MTBE fraction. Other compounds present in significantly lower concentrations were protocatechuic acid, vanillin, coniferyl alcohol, syringaldehyde, and sinapic acid. Thus, the major phenolic compounds in the ether extract are the result of the acid degradation of tannin and lignin.

The degradation of lignin during acidolysis is mainly due to the cleavage of the ether bonds; during dilute acid treatment the β-*O*-4 end units of lignin are preferentially cleaved. After cleavage of this bond, the released phenolic can undergo rearrangement reactions to generate various low-molecular-weight compounds. For example, some phenolic compounds identified in wood hydrolyzates and lignin acidolysis mixtures include vanillin, coniferylaldehyde, syringaldehyde, vanillic acid, and syringic acid (*25, 26*). The hydrolyzable tannins of oak will decompose forming gallic acid, ellagic acid, and glucose. Several of the compounds described above were also reported by Tran and Chambers to be present in an extractive-free oak-prehydrolyzate stream, and also found to be inhibitory to *Pichia stipitis* (*13*).

Analysis of the Extractive-Free Biomass Samples

The extractive free-biomass of red oak, white oak, yellow poplar and treated solids were subjected to summative biomass analyses (*16*), and the values are given in Table 2. Glucose and xylose were the only carbohydrates quantified. In this protocol, a correction is made based on the assumption that all carbohydrates destroyed during the acidolysis process are converted to either HMF or

furfural. Thus, any decomposition of sugars should be quantified by the HMF and furfural values, and these values can be added to glucose and xylose values, respectively. The hexosan and pentosan values are corrected for mass changes associated with hydrolysis. The method employed to estimate the percent biomass does not account for acetate, uronic acids and the other hemicellulosic and pectic sugars. Therefore, the values are less than 100%. The ash contents reported are probably higher than the true values due to the high degree of error associated with the gravimetric determinations (ash contents were determined from 100 mg of starting material). The ash contents for oak and yellow poplar are on the order of 0.1 to 1.3% and 0.3% (dry mass basis), respectively (*10*). The ash content of the treated solids was negligible, since the acid pretreatment solubilized the inorganics.

Toxicity Testing of the Hydrolyzate against *Zymomonas mobilis*

The goal of this study was to investigate the relative toxicity of hydrolyzate fractions at various dilutions toward *Zymomonas* cell growth and ethanol production. All the fractions and controls prepared were supplemented with glucose and xylose to provide concentrations matching that of the original hydrolyzate, and these toxic effects were compared to a "positive" control, which functions as a pure sugar control. Initial studies were concerned with characterization of the positive control relative to overlimed and direct neutralized hydrolyzate fractions at various loading levels. A 100% loading level represents sugar concentrations in bioassay tubes which match that of the original hydrolyzate, and lower percentage loading values represent the corresponding lower hydrolyzate concentrations. For example, a 30% level represents a bioassay (direct neutralized or overlimed), which contains 3 parts hydrolyzate and 7 parts DI water. The overlimed hydrolyzate is currently considered the "best-case scenario" for industrial processing as previous studies have indicated that treatment of hydrolyzate liquors with strong bases helps reduce inhibition (*11*).

All hydrolyzate bioassays were performed at 30% loading levels since precipitation in the bioassay tube increased with increased hydrolyzate levels. The relative toxicity studies were carried out by measuring the OD_{600nm} of the cultures to determine cell growth, and by ethanol yield

to ascertain fermentation performances. The net OD_{600nm} values, and ethanol yields for positive and overlimed controls as well as for the neutralized 30% hydrolyzate are shown in Table 3. All values are averages of duplicate samples.

These data reveal inhibition of cell growth and ethanol production at the 30% level. The increased OD_{600nm} value for the 30% (v/v) hydrolyzate level relative to the negative control is attributed to the turbidity caused by precipitation that tended to cling to the sides of the tubes. In support of this statement are the data obtained from the HPLC analysis of the supernatant, which indicate a lower ethanol yield for the 30% hydrolyzate (v/v) relative to the overlimed negative control (0.6 mg/mL vs. 2.37 mg/mL, respectively).

Two important facts were revealed from this experiment: First, the precipitation caused by the hydrolyzate makes the OD values for ascertaining cell growth somewhat unreliable. Although these nonlinear OD measurements have been corrected with an appropriate blank, when precipitation takes place the method should be used only for obtaining a rough estimate of the cell growth of organisms in a specific medium. Second, based on the ethanol yields, the hydrolyzate clearly inhibits the ability of the recombinant *Zymomonas* to ferment xylose to ethanol, and can only be marginally improved by overliming protocols.

Inhibitory Effects of Suspected Toxins

In order to determine the most toxic components in the liquid hydrolyzate, the data obtained from the HPLC analysis of the liquid hydrolyzate were used to estimate the loading levels for bioassays performed with individual suspected inhibitors (see Table 4). In addition to compounds detected in the hydrolyzate, toxic effects of several other potential inhibitors (*13*), including the alkaloid glaucine (a major compound present in yellow poplar), were analyzed. Three loading levels were chosen for each compound tested. No precipitation was observed during the addition of pure compounds to the test media, and thus cell growth efficiency was evaluated from OD values at all three loading levels. Ethanol yields were measured at the 100%, and the results are given in Table 4.

In general, the cell growth values (OD) followed that of the ethanol yields. Inconsistent results from the cell growth inhibition studies preclude their complete presentation herein. While complete explanation of the observed inconsistencies is not possible, part of the problem may reside with the organism itself and its response to the compounds added. For example, visualization of the furfural and HMF bioassay samples under the microscope revealed that the organisms responded differently to the two compounds. In the presence of HMF the cells remain viable but tend to agglomerate, modifying the OD response. These results clearly suggest that care must be taken in relying on growth measurements (OD_{600nm}) in assessing the toxicity of hydrolyzate components. The only completely reliable method of ascertaining fermentation efficiency is through determination of ethanol yields.

The ethanol yields indicate that acetic acid is by far the most toxic compound, followed by caproic acid and furfural (Table 4). Except for vanillin and syringaldehyde, the other phenolic compounds tested have only a moderate effect on ethanol production. Most of the compounds are therefore only slightly inhibitory to cell growth and ethanol production, with organic acids being the strongest inhibitors. There is also a trend in which organic acids and aldehydes are more inhibitory than lignin acids, alcohols, or the one alkaloid tested. These results suggest that efforts to minimize toxicity should initially be directed at removing the acetic acid formed during the acid hydrolysis.

Toxicity Testing of Extracts from Treated and Untreated Solid Biomass Samples

Lignin and extractives in wood can be solubilized during the acid pretreatment of woody biomass, without formation of compounds such as acetic acid. These extracts may or may not be toxic to the organisms that convert sugars to ethanol. However, as a major role of extractives in a growing tree is to prevent pathogen invasion, the compounds are inherently toxic. Oaks contain a high concentration of hydrolyzable tannins, whereas yellow poplar is noted for its alkaloid content (*22,24*). Therefore, the toxicity of these fractions should be ascertained, as species with highly toxic extracts may not be desirable as substrates for industrial bioconversion.

Due to the poor solubility of the extractives either in ethanol or ethanol:water, and the severe precipitation that occurred when these extractives were added to the media, testing was performed at 30%, 15%, and 5% loading levels (see Table 5 for details on estimating of loading levels). Even at these levels, adding extractives to media tended to cause precipitation, thereby limiting the usefulness of OD measurements.

The results indicate that the red oak and white oak acetone:water extracts are more toxic than the methanol extracts toward *Zymomonas* ethanol fermentation. In both instances, white oak extracts were the most toxic, and yellow poplar extractives were the least toxic. These results correlate with what is known about wood and its resistance to microbial decay. Of the three species, white oak is the most durable, followed by red oak and yellow poplar. This resistance to decay is mostly due to the tannins present in the white and red oaks. The mechanism of biological resistance is not completely clear, but tannins are well known for their ability to precipitate proteins (*29*). Extracellular degradatory enzymes secreted by a wood-invading organism would, when in intimate contact with a tannin molecule, undergo precipitation and subsequent inactivation (*24*). Thus, it is also understandable that significant precipitation occurred in the growth inhibition bioassay.

The oak extractives being much more inhibitory than that of yellow poplar suggests that yellow poplar or fast-growing wood species such as cottonwood, aspen, or poplar (low tannin content woods) would be much more desirable substrates for bioconversion. Our future studies will concentrate on a hydrolyzate liquor prepared from yellow poplar sawdust.

Removing of compounds from wood hydrolyzates that are inhibitory to microorganisms should improve the overall efficiency of the biomass conversion process. Previous studies indicate that treating wood hydrolyzate with sodium hydroxide, calcium hydroxide, or a strong anionic exchange resin helps remove these compounds (*11, 27*). The results obtained here suggest that with respect to xylose utilization, the increase in fermentation efficiency is low when overliming is used. A qualitative ranking for the compounds and extracts tested is shown in Figure 2. Acetic acid and the oak extracts were the most inhibitory compounds in the liquid hydrolyzate, furfural

exhibited a moderate effect. Solvent extraction methods such as this may be useful in separating a fairly toxic organic fraction from the liquid hydrolyzate, and this should be explored in more detail. For example, a wood-to-ethanol facility may be able to use ethyl acetate as the extraction solvent with the ester made from the ethanol and acetic acid produced at the facility. This and other amelioration strategies are currently under investigation.

ACKNOWLEDGMENTS

This work was funded by the Biochemical Conversion Element of the Office of Fuels Development of the US Department of Energy through subcontract XAC-4-13363-01with the National Renewable Energy Laboratory (NREL), Golden, CO. Ekaterina Martinson and Ulli Becker are gratefully acknowledged for their technical assistance. The authors are also grateful to the Department of Chemistry, Virginia Tech for use of the GC/MS.

REFERENCES

1. Lawford, H. G. (1988), *Appl. Biochem. Biotechnol.* **17**, 203-219.

2. Baratti, J., and Bu'Lock, J. D. (1986), *Biotechnol. Adv.* **4**, 95-115.

3. Rogers, P. L., Lee, K. J., Skotnicki, M. L., and Tribe, D. E. (1982), *Adv. Biochem. Eng.* **23**, 27-84.

4. Lee, K. J., Tribe, D. E., and Rogers, P. L. (1979), *Biotechnol. Lett.*, **1**, 421-426.

5. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., and Picataggio, S. (1995), *Science*, **267**, 240-243.

6. Keim, C. R. (1983), *Enzyme Microbiol. Technol.* **5**, 103-114.

7. Rodriguez, E., and Callieri, D. A. S. (1986), *Biotechnol. Lett.*, **8**, 745-748.

8. Beavan, M., Zawadzki, B., Droniuk, R., Lawford, H., and Fein, J., (1989), *Appl. Biochem. Biotechnol.*, **20/21**, 319-326.

9. Parekh, S. R., Parekh, R. S., and Wayman, M. (1989), *Process Biochem.*, **24**, 88-91.

10. Pettersen, R. C. (1984), *ACS Symp. Ser.*, **207**, Washington D.C., 57-126.

11. McMillan, J. D. (1994), *ACS Symp. Ser.*, **556**, Washington D. C., 411-437.

12. Ingram, L. O., Alterthum, F., Ohta, K., and Beall, D. S. (1990), *Developments in Industrial Microbiol.*, **31**, 21-30.

13. Tran, A. V., and Chambers, R. P. (1986), *Enzyme Microb. Technol.*, **8**, 439 -444.

14. Örsa, F., and Holmbom, B. (1994), *J. Pulp and Paper Sci.*, **20**, 361-366.

15. Kermasha, S., Goetghebeur, M., and Dumont, J. (1995), *J. Agric. Food Chem.*, **43**, 708-716.

16. Kaar, W. E., Cool, L. G., Merriman, M. M., and Brink, D. L. (1991), *J. Wood Chem. Technol.*, **11**, 447-463.

17. Morris, W. E. and Johnson, D. B. (1967), in *Procedures for the Chemical Analysis of Wood Products*, USDA Forest Products Lab, Madison, WI.

18. *Technical Association of the Pulp and Paper Industry (TAPPI) Standard Methods*, T13, T222, T250, and T211.

19. Antal, M. J., Leesomboon, T., Mok, W. S., and Richards, G. N. (1991), *Carbohydr. Res.*, **217**, 71-85.

20. Timell, T. E. (1964), *Adv. Carbohydr. Chem.*, **19**, 247-302.

21. Fengel, D. and Wegener, G. (1989) *Wood: Chemistry, Ultrastructure, Reactions*, Walter de Gruyter, Berlin, 2nd ed, p. 274.

22. Rowe, J. W. and Conner, A. H., (1979), in *Extractives in Eastern Hardwoods, A Review*, Gen. Tech. Rep., FPL-18, USDA Forest Products Laboratory, p. 67.

23. Hillis, W.E. (1987), in *Heartwood and Tree Exudates*, Springer-Verlag, Berlin, 268p.

24. Scalbert, A. (1992), in *Plant Polyphenols*, Hemingway, R. W.; Laks, P. E., ed., Plenum Press, New York, 259-280.

25. Frazer, F. R. and McCaskey, T. A. (1989), *Biomass*, **18**, 31 -42.

26. Lapierre, C., Rolando, C., and Monties, B. (1983), *Holzforchung*, **37**, 189-198.

27. Stanek, D. A. (1958), *TAPPI*, **41**, 601-609.

28. McCaskey, T. A., Rice, M. D., and Smith, R. C. (1986), in *Biomass Energy Development*, Smith, W. H., ed., Plenum, New York, 573-585.

29. Luck, G., Liao, H., Murray, N. J., Grimmer, H. R., Warminski, E. E., Williamson, M. P., Lilley, T. H., and Haslam, E., (1994) *Phytochemistry*, **37**, 357-371.

Table 1. Extractive Yields (mass percent on a dry basis).

Sample	Acetone:Water	Hot Methanol	Total Extracts	% Extractives[a]
Yellow poplar	1.51	1.39	2.90	2.4
Red oak	5.36	2.30	7.66	4.4-9.6
White oak	2.42	4.33	6.75	5.3-6.6
Treated solids	8.67	1.95	10.12	-

[a] Values taken from Ref. 10.

Table 2. Summative Analysis of the Biomass Samples.

Component (%)	Red oak	White oak	Yellow-poplar	Treated solids
Hexosan[a]	35.20	36.51	38.99	53.28
Pentosan[b]	16.70	17.70	14.53	0.02
Acid soluble lignin	3.14	3.49	3.48	1.14
Klason lignin	24.50	24.50	23.0	28.0
Ash	0.8	2.0	1.2	0.0
Moisture (105 °C)	7.99	7.46	7.59	8.99
Total[c]	94.5	98.1	95.0	97.3

[a]Corrected for mass change according to the eqn.: Hexosan = (glucose)(0.9) + (HMF + 162/110).
[b]Corrected for mass change according to the eqn.: Pentosan = (xylose)(0.88) + (furfural + 132/96).
[c] Total = %Hexosan + %Pentosan + %Klason lignin + %Acid-soluble lignin + %Ash + %Moisture. This total does not account for acetate, uronic acids or other hemicellulosic/pectic sugars.

Table 3. Net OD_{600nm} Values and Ethanol Fermentation Yields for the Positive Control and the Overlimed and Direct Neutralized Hydrolyzates.

Sample[a]	Net OD_{600nm}	Ethanol (mg/mL)	% Performance[b]
Positive Control	1.051	18.70	100
Overlimed Hydrolyzate (30% loading)	0.587	2.37	13
Direct Neutralized Hydrolyzate (30% loading)	0.999	0.60	3

[a]The initial amounts of xylose and glucose in samples are 44.7 mg/mL and 2.4 mg/mL, respectively. Fermentation time was 72 h.
[b]Calculated by the equation: [(Final EtOH conc. sample)/(Final EtOH conc. positive control)]100.

Table 4. Ethanol Fermentation Yields Obtained after a 48-h Fermentation with Individual Suspected *Zymomonas* Inhibitors (100% Level).

Sample	Inhibitor Concentration[a] (mg/mL)	Ethanol[b] (mg/mL)	% Performance
Positive control	-	13.43-14.83	100
Overlimed hydrolyzate	-	1.37 - 1.43	9
Acetic acid	9.03	>1	0
Furfural	0.95	8.52	58
HMF	0.09	11.86	80
Gallic acid	0.173	11.39	77
Syringaldehyde	0.130	9.48	64
Coniferyl alcohol	0.050	12.10	82
Vanillin	0.043	8.71	65
Vanillic acid	0.084	13.63	101
Sinapic acid	0.060	11.93	89
Syringic acid	0.093	12.80	95
Protocatechuic acid	0.050	9.68	72
Glaucine	0.052	10.13	75
Caproic acid	0.064	7.64	57

[a] Inhibitor concentrations used in this study were estimated from the HPLC analyses of compounds detected in the hydrolyzate. Inhibitor concentrations for other compounds were based on the values from reference (13). The glaucine level was estimated based upon extractive content of yellow poplar, assuming that glaucine was 10% of the extract.
[b] Samples were added to media dissolved in 0.1 mL of ethanol. Thus, the ethanol yields reported are subtracted from the initial ethanol in the media.

Table 5. Ethanol Fermentation Yields after 48 h for the Controls and Wood Extracts.

Sample (% Loading)[a]	Ethanol (mg/mL)[b] Acetone:Water Extract (% Performance)	Ethanol (mg/mL) Hot Methanol Extract (% Performance)
Positive control	16.79 (100)	16.79 (100)
Overlimed hydrolyzate	1.12 (7)	1.12 (7)
Red oak (30%)	1.26 (8)	7.23 (43)
Red oak (15%)	1.40 (9)	12.47 (74)
Red oak (5%)	6.81 (41)	11.87 (71)
White oak (30%)	>1 (0)	>1 (0)
White oak (15%)	1.32 (8)	>1 (0)
White oak (5%)	4.71 (28)	3.51 (21)
Yellow poplar (30%)	11.26 (67)	11.33 (67)
Yellow poplar (15%)	13.72 (82)	12.87 (77)
Yellow poplar (5%)	11.86 (70)	12.43 (74)
Treated solids (30%)	2.82 (17)	13.76 (82)
Treated solids (15%)	10.03 (60)	14.60 (87)
Treated solids (5%)	13.59 (81)	12.03 (72)

[a]The levels of extractives added to the media were estimated based on the extractive yields obtained from the samples, and the amount of xylose found in the wood hydrolyzate using the relationship: (% yield of extract)/(% xylose in wood) = (extract conc. in hydrolyzate)/(xylose conc. in hydrolyzate). This represents the relationship between extractive and xylose contents, if one assumes that all of the extractives and xylose were solubilized by the pretreatment process.

[b]All the extracts were freeze-dried to ensure complete removal of solvents, and the extractives were added to the media dissolved in ethanol:water (1:1, 0.2 mL). Ethanol values reported are values obtained after subtracting initial ethanol in media (at T_0).

FIGURE CAPTIONS

Figure 1. HPLC Separation of the MTBE Extract. Conditions are as described in the text. Compound Key: **1**, gallic acid; **2**, HMF; **3**, furfural; **4**, protocatechuic acid; **5**, unknown; **6**, vanillin; **7**, coniferyl alcohol; **8**, syringaldehyde; **9**, sinapic acid.

Figure 2. Relative Compound/Extract Inhibition Scale for *Zymomonas mobilis* CP4(pZB5) Xylose Fermentation.

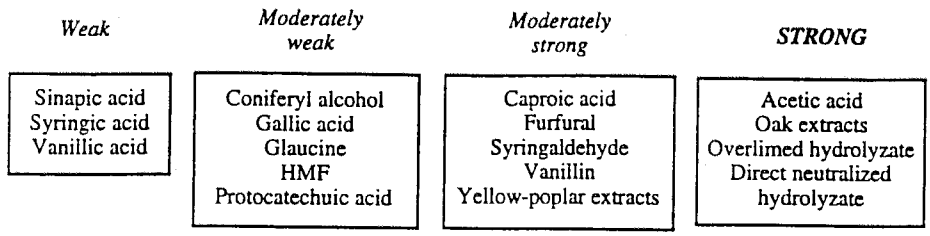

FIGURE 2. Ranatunga et al.

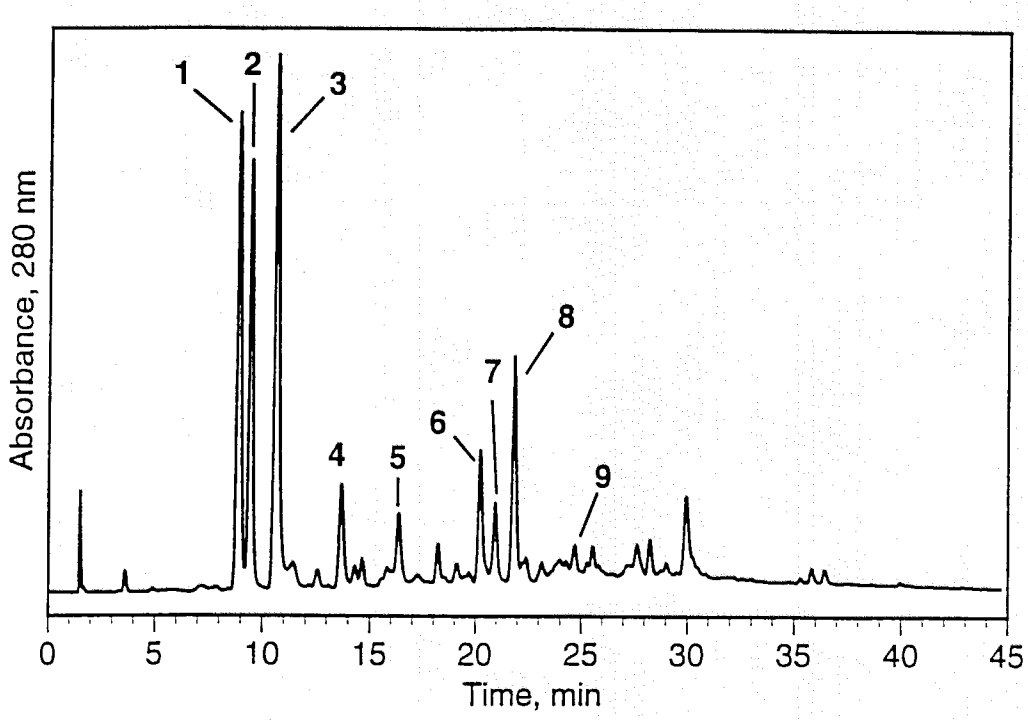

FIGURE 1. Ranatunga et al.

Appendix C—Preliminary Technical Report Update

This Appendix is provided as an attachment to the NREL Final Technical Report (Subcontract XAC-4-13363-01), and discusses the work performed during the period of 01 February 1997-15 March 1997.

Zymomonas mobilis Cp4(pZB5) Bioassays using Shake Flasks and pH Controlled Media. Our previous bioassay experiments were performed on a 10 mL-scale in test tubes (12 mL) without any pH control.[1] Recent discussions with NREL have indicated that better performance and reproducibility can be achieved utilizing larger shake flasks as well as pH control. As the use of pH control will probably be part of the new LAP for bioassays, efforts were taken to develop the protocol for *Zymomonas mobilis* Cp4(pZB5) bioassay experiments in our laboratory.

The experiments were run for either 24 or 48h, and the pH was determined and modified at 3, 6, and 24 h time intervals. The target pH during the adjustment was 5.8-6.0 with autoclaved 2M KOH. Our initial experiments were carried out in baffle flasks (250 mL) containing 60 mL of positive control (Glucose - 6.38 g/L, Xylose - 35.88 g/L, Yeast extract 10 g/L, KH_2PO_4 - 2 g/L, and tetracycline) or negative control (30% level of the NREL hydrolyzate P961014 supplemented with sugars) media. The flasks were inoculated with *Zymomonas* to achieve an initial OD of 0.2, sealed with aluminum foil, and incubated at 30°C at 90 rpm. The ethanol yields were compared with positive control flasks without pH adjustment as well as with positive control test tube bioassays.

pH adjustment is made as follows. An aliquot of fermenting broth (2 mL) is removed from the shake flask and assayed for pH (Accumet meter with a small electrode). Once the pH is determined, the amount of base to be added to the remaining contents of the shake flask is determined. Once the addition is made, the pH of the shake flask is checked again by removing an aliquot of broth. After a bit of practice, we were able to almost always add the appropriate amount of base the first time.

After a 48 h incubation, the ethanol yields in the flask setups were much lower than those obtained from the test tube setups. HPLC analyses of the pH-controlled flasks indicated that only about a 48% of xylose had been utilized after 48 h, whereas the flasks without pH control had only 20% xylose utilization. Thus while ethanol performance was poor in the shake flasks, it was observed that the pH adjustment did help xylose utilization.

To improve the shake flask fermentation efficiency, several hypotheses were developed to rationalize the low ethanol yields. Our first hypothesis was loss of ethanol by evaporation—use of flasks sealed with aluminum foil may not only allow for CO_2 release, but ethanol release as well. The possibility of ethanol

evaporation was evaluated by mixing media with ethanol in the shake flask and exposing them to conditions exactly the same as those used in the bioassays. No significant ethanol loss was noted. Nonetheless, in accordance to the procedures utilizied at NREL, our shake flasks are now rubber-stoppered with a glass tube inserted through the stopper and plugged with glass wool.

Our second hypothesis was that the flasks werebeing shaken too violently. Thus, the next set of experiments were carried out in regular 250 mL Erlenmeyer flasks (ie., no baffles) containing 60 mL of media, sealed with rubber stoppers containing glass wool plugged vent tubes, at 30^{0}C at a speed of 40 rpm. After these modification, the ethanol yields increased up to an average of 17.35 mg/mL in the pH controlled flasks during 24 h. The average ethanol yield of the reference positive control (pH not adjusted) flasks were 14.17 mg/mL. These experiments were repeated using media containing low phosphates (0.2 g/L), and the average ethanol yields obtained from the pH controlled flasks was 11.44 mg/mL.

At this stage we are still concerned with the reproducibility of ethanol yields from the shake flask method, as they vary on the order of 2-3 mg/mL. Thus, our efforts are still focused on optimizing the conditions for the pH controlled shake flask method to obtain maximum ethanol yields and reproducibility.

Studies on Uronic Acid Contents of Hydrolyzate Samples. Preliminary studies were carried out to develop an assay to determine the uronic acid content of hydrolyzate samples. This study was initiated to understand whether uronic acid content has any effect on the toxicity of various hydrolyzates. Although several spectrophotometric methods have been reported to monitor uronic acids, in many instances these methods are compromised by interfere with the sugars, furans, and phenolics present in the samples.[2-4] A more sensitive and specific method developed by Blumenkrantz and Asboe-Hansen has been observed to have less interference from hexoses.[5] The method is based on measuring the absorbance of a colored complex formed when uronic acids are heated to 100 °C with conc. H_2SO_4/tetraborate followed by reaction with *meta*-hydroxy diphenyl (also known as 3-phenylphenol).

As a preliminary investigation, the two yellow poplar hydrolyzate samples (P961014 and P960227SD) were analyzed using this method. The uronic acid contents were estimated as D-glucuronic acid lactone equivalents. A synthetic hydrolyzate containing glucose, xylose, furfural, HMF and acetic acid (in dilute sulfuric acid) was used to verify interference from neutral sugars, furans, and acetic acid. The diluted synthetic hydrolyzate (dil x 250) did not give any significant color reaction. However, when the hydrolyzate (dil x 250) was heated with conc. H_2SO_4/tetraborate solution, a light yellow color formed without addition of 3-phenylphenol. This may be due to interference from phenolic compounds. Although this absorbance was subtracted from the absorbance of a hydrolyzate treated according to assay protocols (conc. H_2SO_4/tetraborate then 3-phenylphenol), non-reproducible results were obtained.

In an attempt to improve the assay system, the hydrolyzate samples were first extracted with MTBE and then subjected to the assay. Table 1 represents the values obtained for the uronic acid content performed in triplicate. These results indicate that uronic acid level in the P961014 hydrolyzate is higher than that of P960227SD. However, we are still somewhat unsure of the capabilities and possible contamination problems associated with the spectrophotometric method. A method is needed to first separate the uronic acids from the hydrolyzate; this fraction will allow us to precisely determine the uronic acid content in hydrolyzates either by spectrophotometric or HPLC methods. We will also be able to ascertain if any non-specific reactions are occuring with hydrolyzate using the spectrophotometric method. We are currently working on an ion exchange procedure to purify the uronic acids from the hydrolyzate samples.

Table 1. Uronic acid Content[a] in Hydrolyzate Samples.

Hydrolyzate P960227SD (mg/mL)	Hydrolyzate P961014 (mg/mL)
1.54 (40%)[b]	3.88 (100%)
1.71 (62%)	2.78 (100%)
1.16 (51%)	2.27 (100%)
1.47 ± 0.28 (49%)	2.98 ± 0.82 (100%)

[a]Expressed as D-glucuronic acid lactone equivalents, values given are obtained from three different assays.[b] Percent values are expressed relative to the NREL Hydrolyzate P961014.

REFERENCES

1. NREL Chemical Analysis and Testing Procedure: Experimental Protocols for Assessing the Toxicity of Hemicellulose Hydrolyzates Using *Zymomonas mobilis* CP4(pZB5) and *Saccharomyces cerevisiae.*

2. Scott R. W. Colorimetric Determination of Hexuronic Acids in Plant Materials, *Anal. Chem.* **51**(1979), 936.

3. Englyst, H. N.; Quigley, M. E.; Hudson, G. J. Determination of Dietary Fiber as Non-starch Polysaccharides with Gas-Liquid Chromatographic, High performance Liquid Chromatography or Spectrophotometric Measurement of Constituent Sugars, *Analyst,* **119**(1994), 1497.

4. Taylor, K. A.; Buchanan-Smith J. G. A Colorimetric Method for the Quantitation of Uronic Acids and a Specific Assay for Galacturonic Acid, *Anal. Biochem.* **201**(1992), 190.

5. Blumenkrantz N.; Asboe-Hansen, G. New method for Quantitative Determination of Uronic Acids, *Anal. Biochem.* **54**(1973), 484.

Appendix D—Saccharomyces Manuscript

Appendix D is a modified version of a manuscript originally submitted as part of Progress Report 11. This manuscript has undergone some serious revision and summarizes some work performed with *Saccharomyces cerevisiae D5A*. We anticipate submitting this article to *Biotechnol. Letters*. We are requesting at this time that the manuscript proceed through the standard NREL editorial process.

TOXICITY OF HARDWOOD EXTRACTIVES TOWARD *SACCHAROMYCES CEREVISIAE* D5A GLUCOSE FERMENTATION

Thilini D. Ranatunga, Judith Jervis and Richard F. Helm
Fralin Biotechnology Center, Department of Wood Science and Forest Products, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0346

James D. McMillan and Christos Hatzis
Biotechnology Center for Fuels and Chemicals, National Renewable Energy Laboratory, Golden, CO 80401-3393

SUMMARY

Few studies have directly addressed the toxicity of wood extractives on the fermentation efficiencies of ethanol-producing organisms. The toxicity of several wood extracts as well as a first-stage hardwood hydrolyzate liquor prepared from a red oak:white oak:yellow poplar (1:1:1) sawdust on *Saccharomyces cerevisiae D5A* has been examined. Acetone:water and hot methanol extracts of solid biomass samples from white oak, red oak, and yellow poplar are moderately toxic to glucose fermentation. The organism exhibits tolerance for the compounds present in the xylose-rich hydrolyzate, with overliming followed by thermal conditioning significantly improving the overall fermentation efficiency.

INTRODUCTION

Numerous studies have been carried out on the bioconversion of woody biomass to fuel ethanol via a two-stage process.[1-7] During this process, hemicellulose and cellulose are hydrolyzed separately to fermentable sugars.[8] For hardwood and most agricultural biomass substrates, the initial pretreatment results in deacetylation and depolymerization of the hemicelluloses producing the pentose sugar, xylose as the major product. This xylose-rich stream is separated from the solids and fermented to ethanol while the solids are submitted to a simultaneous saccharification and fermentation process for the conversion of the available glucose.

During the first-stage dilute acid pretreatment of hardwoods, undesireable reactions such as dehydration of

xylose to furfural can occur.[9] In addition, lignin and extractives present in wood can be solubilized during the treatment process or undergo acid-catalyzed degradation reactions. These compounds can affect the efficiency of the bioconversion process and ultimately compromise ethanol yields.[10-13]

While numerous studies have been performed assessing the toxicity of lignin degradation products, furans and organic acids,[10-12, 14] few studies have directly assessed the role extractives play in ethanol fermentation efficiencies. As one of the major functions of extractives in woody plants is to protect the living tree from pathogen invasion, it seems somewhat reasonable to assume that they may also be toxic to ethanol producing organisms. A previous investigation on extractives from red oak, white oak, yellow-poplar and mixed hardwood fibers indicated that oak extracts were highly toxic to *Zymomonas mobilis* CP4(pZB5) xylose fermentation.[12] It was further suggested that hardwoods such as the oaks may be somewhat undesireable species due to the inherent toxicity of their extracts.[12]

MATERIAL AND METHODS

General. Optical density (OD) measurements were obtained at 600 nm with a Milton-Roy Spectronic 601 spectrophotometer (slit width ~ 5 mm). All media used were pH adjusted to 5.0 with phosphoric acid, and autoclaved for 20 min at 121 °C. *S. cerevisiae* D5A was obtained from the National Renewable Energy Laboratory (NREL), Golden, CO. The inoculum preparation procedure involved two stages. During the first stage, a thawed stock vial of *S. cerevisiae* was inoculated into a 50 mL of sterile YPD medium (yeast extract 10 g/L, peptone 20 g/L, D-glucose 20 g/L) in a 250-mL baffled flask. The flask was incubated at 38°C and 90 rpm for 8-12 h. In the second stage, the YPD grown culture was transferred to a flask containing 10 mL of YP medium (yeast extract 100g/L, peptone 200 g/L), D-glucose (500g/L, 10 mL), seed culture from the first stage flask (10 mL), and DI water (70 mL). The flask was incubated at 38°C and 90 rpm for 8-12 h.

All the toxicity studies were carried out in sterile screw-capped test tubes (12 mL) containing 10 mL of "positive control" medium (yeast extract 10 g/L, peptone 20 g/L, D-glucose 40 g/L). Extracts were added to the tubes dissolved in ethanol:water (1:1, 0.2 mL) and each tube was then inoculated with *Saccharomyces* cells to achieve an optical density (OD_{600nm}) of 0.1. The tubes were incubated at 38 °C in a shaker for 48 h, at which time cell growth was ascertained by measuring the nonlinear OD of the cultures at 600 nm. The non-linear OD measurements for each tube was corrected with an appropriate blank (without *Saccharomyces* cells), and the net OD values were obtained by subtracting the OD of test sample from the OD of medium blank. Toxic effects were also monitored by determination of ethanol yields after 48 h.

Substrate Preparation. Acetone:water and hot methanol extracts of red oak, white oak and yellow poplar

were obtained as described previously.[12] The red oak:white oak:yellow poplar (1:1:1) first-stage mixed hardwood hydrolyzate was prepared according to a previously described method.[12] The direct neutralized hydrolyzate was prepared by neutralizing the original hydrolyzate liquor (pH 1.1) with $Ca(OH)_2$ to achieve a final pH of 6.0. The solution was then filtered through a 0.2 μm sterile filter, and stored at 4 °C. The overlimed hydrolyzate was prepared by adjusting the pH of the hydrolyzate liquor to pH 10.0-10.5 with solid $Ca(OH)_2$. The mixture was heated at 50 °C for 30 min, and the pH was adjusted to 7.0 using 96% (w/w) H_2SO_4. The liquor was filtered through a 0.2 μm sterile filter, and stored at 4 °C for minimum of 5 d. On the day of the experiment, a 30% level of direct neutralized/overlimed hydrolyzate fraction was prepared for toxicity testing by mixing yeast extract (1 g), peptone (2 g), glucose (4 g), direct/overlimed hydrolyzate (30 mL), and DI water (70 mL).

Ethanol Determinations. The ethanol determinations were carried out using a Varian Gas Chromatograph equipped with a flame ionization detector, and a Porapak Q 80/100 glass column (2m x 4mm i.d) packed in-house. Isopropanol was used as the internal standard. The detector and injector temperatures were 250 °C and 230 °C, respectively. The column temperature was programmed from 175 °C to 180 °C at 2 °C/min.

RESULTS AND DISCUSSION

Toxicity Testing of the Extractives from Solid Biomass Samples. The extractives were prepared according to a previously described method, and all samples were freeze dried prior to toxicity testing to ensure complete removal of extraction solvents.[12] The extracts were tested at three levels with severe precipitation observed during addition of extractives to the media at high loading levels. Even at low addition levels precipitation was noted, thereby limiting the usefulness of OD measurements for monitoring cell growth. Table 1 illustrates the ethanol fermentation yields for extractives at various levels. No major differences in ethanol yields were observed with the acetone:water and methanol extracts. However, none of the extracts were extremely inhibitory to D5A at a 60% level, the highest level tested. These results are in contrast to those obtained with *Zymomonas mobilis* CP4(pZB5), where almost complete inhibition was observed at 30% loading levels for the oak extracts.[12]

Toxicity Testing of Hydrolyzate Fractions. Investigations were also carried out on various hydrolyzate fractions to evaluate the ethanol producing efficiency of D5A. The initial glucose concentration of these fractions were maintained at 40 g/L, and the toxic effects were compared to a "positive control" which functions as the pure sugar control. The toxicity experiments were carried out on 30% and 60% loading levels of the direct neutralized samples, and at a 30% level of the overlimed hydrolyzate. A 30% or 60% loading level represents a direct neutralized/overlimed hydrolyzate (30 or 60 mL) fraction supplemented

with an initial concentration of glucose (40 g/L), and diluted to 100 mL with DI water.

Table 1. Ethanol Yields after 48-h Fermentations With Several Wood Extracts.

Sample	% Loading[a]	Ethanol (mg/mL) Acetone:water extracts[b]	Ethanol (mg/mL) Methanol extracts[c]
Positive control[c]		18.59 (100)	18.59 (100)[d]
Red oak (RO)	60	17.12 (92)	insol.[d]
	30	17.32 (93)	17.30 (93)
	15	17.06 (92)	16.95 (91)
White oak (WO)	60	16.28 (88)	insol.
	30	16.26 (87)	17.06 (92)
	15	17.80 (96)	16.52 (89)
Yellow poplar (YP)	60	16.89 (91)	insol.
	30	16.37 (88)	16.99 (91)
	15	17.57 (95)	17.44 (94)

[a]The levels of extractives added to the media were estimated based on the extractive yields obtained from the samples, and the amount of xylose found in the wood hydrolyzate using the relationship: (% yield of extract)/(% xylose in wood) = (extract conc. in hydrolyzate)/(xylose conc. in hydrolyzate). This represents the relationship between extractive and xylose contents, if one assumes that all of the extractives and xylose were solubilized by the pretreatment process. See Ref. 12 for hydrolyzate and wood compostions as well as extract yields. [b]Ethanol yields reported are subtracted from the initial ethanol content in the media. Values in parentheses are the percent performance values relative to the ethanol control[c]A positive control with 0.2 mL of ethanol:water (1:1) mixture. [d]Sample was insoluble at this concentration.

The OD values and ethanol yields obtained with these fractions are given in Table 2. A 60% level of the direct neutralized hydrolyzate was inhibitory to the cell growth and glucose fermentation of *Saccharomyces* (percent performance 61%). This implies that the first-stage hydrolyzate contains compounds that are toxic to D5A. Overliming has improved the fermentation performances at a 30% level and is probably effective in removing some of the inhibitory hydrolyzate components. A first-stage hardwood hydrolyzate treated with calcium hydroxide has been observed to have reduced toxic effects,[15] and although the mechanism of action is unknown, treatment of hydrolyzate liquors with strong base is helpful in reducing inhibition.[10, 13]

Table 2. Cell Growth and Ethanol Yields after 48-h Fermentations with Various Hydrolyzates.

Sample (% Level)	Net OD_{600nm}			Ethanol (mg/mL)	Percent
	T_0	T_{24}	T_{48}	T_{48}	Performance
Positive control	0.093	1.90	1.92	17.7	100
Overlimed Hydrolyzate 30% Level	0.087	1.48	1.46	17.3	97
Neutralized Hydrolyzate 60% Level	0.300	0.32	0.61	10.8	61
Neutralized Hydrolyzate 30% Level	0.068	0.36	0.88	15.8	89

CONCLUSION

From the bioassays it is evident that *Saccharomyces cerevisiae* D5A is tolerant of the inhibitory compounds present in a first-stage hydrolyzate liquor as well as wood extracts. This suggests that, at least for glucose fermentation, hardwood extracts are only moderately compromising fermentation efficiencies, and this effect can be ameliorated through standard overliming protocols.

ACKNOWLEDGMENTS

This work was funded by the Biochemical Conversion Element of the Office of Fuels Development of the US Department of Energy through subcontract XAC-4-13363-01 with the National Renewable Energy Laboratory (NREL), Golden, CO. Ekaterina Martinson is gratefully acknowledged for her technical assistance.

REFERENCES

1. South, C. R.; Hogsett, D. A., and Lynd, L. R. , Continuous Fermentation of Cellulosic Biomass to Ethanol. *Appl. Biochem. Biotechnol*, **39/40** (1993), 587-600.

2. Beavan, M.; Zawadzki, B,; Droniuk, R.; Lawford, H.; Fein, J. Comparative Performance Trials with Yeast and *Zymomonas* for Fuel Alcohol Production from Corn. *Appl. Biochem. Biotechnol.*, **20/21** (1989), 437-448.

3. Gong, C. S.; Chen, C. S.; Chen, L. F., Pretreatment of Sugar Cane Bagasse Hemicellulose Hydrolyzate for Ethanol by Yeast. *Appl. Biochem. Bio Technol.*, **39/40** (1993), 83-88.

4. Jeppsson, H.; Yu, S.; Hähn-Hagerdal, B. Xylulose and Glucose Fermentation by *Saccharomyces cerevisiae* in Chemostat Culture. *Appl. Envi. Microbiol.*, **62** (1996), 1705-1709.

5. Olsson, L.; Hähn-Hagerdal, B. Fermentation Performance of Bacteria and Yeasts in Lignocellulose Hydrolyzates. *Process Biochem.* **28** (1993), 249-257.

6. Keim, C. R. Technology and Economics of Fermentation Alcohol-An Update. *Enzyme Microb. Technol.*, **5** (1983), 103-114.

7. Hähn-Hagerdal, B.; Linden, T.; Senac, T.; Skoog, K. Ethanolic Fermentation of Pentoses in Lignocellulose Hydrolyzates. *Appl. Biochem. Biotechnol.* **28/29** (1991), 131-144

8. Prieto, S.; Clausen , E. C.; Gaddy, J. L. Improved Hydrolysis Process for the Saccharification of Biomass. *Proceedings Eighth Symposium on Biotechnology for Fuels and Chemicals*, 1986, 123-133.

9. Antal, M. J.; Leesombon, T.; Mok, W. S.; Richards, G. N. Mechanism of Formation of 2-Furaldehyde from D-Xylose. *Carbohydr. Res.* **217** (1991), 71-85.

10. McMillan, J. D. Conversion of Hemicellulose Hydrolyzates to Ethanol. *ACS Symp. Ser.*, 566, American Chemical Society, Washington D. C. 1994, 411-437.

11. Tran, A. V.; Chambers, R. P. Ethanol Fermentation of Red Oak Acid Prehydrolyzate by the Yeast *Pichia stipitis* CBS 5776. *Enzyme Microb. Technol.*, **8** (1986), 439-444.

12. Ranatunga, T. D.; Jervis, J.; Helm, R. F.; McMillan, J. D.; Hatzis, C. Identification of Inhibitory Components Toxic toward *Zymomonas mobilis* CP4(pZB5) Xylose Fermentation. *Appl. Biochem. Biotechnol.*, (1997), in Press.

13. Beck, M. J. Factors Affecting Efficiency of Biomass Fermentation to Ethanol. *Proceedings Eighth Symposium on Biotechnology for Fuels and Chemicals*, 1986, 617-627.

14. Delgense, J. P.; Moletta, R.; Navarro, J. M. Effects of Lignocellulosic Degradation Products on Ethanol Fermentation of Glucose and Xylose by *Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis*, and *Candida shehatae. Enzyme Microb. Technol.* **19**(1996), 220-225.

15. Frazer, F. R.; McCaskey, T. A. Wood Hydrolyzate Treatments for Improved Fermentation of Wood Sugars to 2,3-Butanediol. *Biomass*, **18**(1986), 439-444.

Appendix E—Attachment to the Final Technical Report

NREL Subcontract XAC-4-13363-01

This Appendix is provided as an attachment to the NREL Final Technical Report (Subcontract XAC-4-13363-01), and discusses the work performed during the subcontract no-cost extension period of 15 March 1997—30 April 1997.

Further Studies on *Zymomonas mobilis* CP4(pZB5) Bioassays using Shake Flasks and pH Controlled Media

The preliminary work carried out on *Z. mobilis* bioassays using shake flasks and pH controlled media were described previously.[1] Although the pH control enhanced the xylose utilization of *Z. mobilis*, we were somewhat concerned about the reproducibility of ethanol yields. Thus, further experiments were carried out to optimize conditions for the pH controlled shake flask method to obtain maximum ethanol yields and reproducibility.

The experiments described previously were carried out in standard 250 mL Erlenmeyer flasks containing 60 mL of media.[1] The maximum ethanol yields obtained from these setups were about 17.4 mg/mL, and the yields varied in the order of 2-3 mg/mL. Considering the high amount of surface area available in these flasks (60 mL media/250 mL flask), we felt that surface aeration may have affected the xylose utilization of *Zymomonas*. This possibility was tested by performing the following experiments.

Bioassays were carried out in 125 mL regular Erlenmeyer flasks containing positive control media (glucose, 6.38 g/L; xylose, 35.88 g/L, yeast extract 10 g/L, KH_2PO_4-2 g/L, and tetracycline). Different volumes of positive control media (120 mL, 90 mL, 60 mL, and 25 mL) were added to each flask, and inoculated with *Zymomonas* to achieve an initial OD of 0.2. The flasks were sealed with rubber stoppers containing glass wool-plugged vent tubes, and incubated at 30 °C at a speed of 50 rpm. The pH adjustments were made as described previously at 3 and 6h.[1] The ethanol yields obtained after 24 h incubation are given in Table 1 with concentrations determined both by GC and HPLC (HPX-87H).

Table 1. Ethanol Yields Obtained after 24 h Fermentation Using Both GC and HPLC.

Flask	Volume (mL)	Ethanol (mg/mL) GC	Ethanol (mg/mL) HPLC
F1	120	19.9	20.0
F2	120	20.4	19.8
F3	90	20.2	19.7
F4	90	20.3	19.6
F5	60	19.0	17.6
F6	25	9.8	8.6

HPLC analysis of the fermentation after 24 hr revealed that maximum xylose utilization was observed in flasks containing either 120 or 90 mL of media (1.38—1.78 mg/mL xylose remained). These results indicate that excessive surface aeration has some adverse effects on xylose utilization. Therefore our future bioassays will use 100 mL of media in 125 mL standard Erlenmeyer flasks (*ie.*, not baffle flasks) to minimize surface aeration, and to obtain maximum ethanol yields.

Studies on Separation of Uronic acid Fraction from the NREL Hydrolyzate P961014

A previous study indicated a higher uronic acid content in NREL Hydrolyzate P961014 relative to P960227SD.[1] This uronic acid content may be partially responsible for the relatively high toxicity of the hydrolyzate P961014. Thus, efforts were made to separate the uronic acid fraction from the hydrolyzate to prepare a fraction which will be useful in toxicity testing. The method employed to isolate the uronic acid fraction was adopted from the method described by Kaar *et al.*,[2] and a detailed description of the method is given in the following section.

A known volume of NREL hydrolyzate P961014 was neutralized to pH 2.5 with a saturated $Ba(OH)_2$ solution. The neutralized solution was centrifuged, and the supernatant was evaporated to a known volume. The solution was then passed through a mixed-bed ion exchange column of Dowex 1X-8 (CO_3^{-2} form) and Bio-Rad AG 50W-X8 (H^+ form) resin. At this point, the uronic acids are expected to be adsorbed to the resin while the neutral sugars are eliminated. To ensure complete removal of all neutral sugars, the column was further eluted with 5-6 bed volumes of water. The eluate was concentrated by rotary evaporation.

The bound uronic acid fraction was then removed by eluting the column with eight bed volumes of 1M acetic acid. The fractions were concentrated by rotary evaporation. Several fractions were collected and analyzed by HPLC using the HPX-87H column with RI detection. The HPLC chromatograms of the acetic acid eluted fractions contained peaks that are believed to correspond with uronic acids, and no peaks corresponding to either xylose or glucose were found. Presence of uronic acids in these fractions were further confirmed by a spectrophotometric assay[3,4] as well as by ^{1}H-NMR. Interestingly, both the neutral and acidic fractions appear to be quite low in acetate content. This would imply that acetylated xylose and xylose oligomers are not present in very high concentrations in the hydrolyzate—acetate cleavage must occur rapidly once oligomers become water soluble.

The HPLC chromatogram of the neutral fraction contained major peaks corresponding to glucose and xylose, and some other peaks which are believed to be either uronic acids or neutral oligomers. NMR

spectra did not provide any strong indications for presence of uronic acids by seaching for the typically strong methyl singlet associated with the 4-*O*-methyl group. Although this fraction provides a color reaction with the spectrophotometric assay[3,4] this may due to some interfering substances present in the material. Further purification and analysis would be needed to prove the presence or absence of uronic acids in this fraction.

Attempts were also made to see whether the separation can be improved by using excess of resin or by using Dowex 1-X4 resin in the acetate form. These modifications did not show any significant differences in the separation pattern. However, from these studies it is apparent that the ion exchange method employed is useful in isolating a major uronic acid fraction from the hydrolyzate. We anticipate performing large-scale ion-exchange purification for isolation of these two fractions as part of the subcontract extension. We would then test for toxicity using shake flask bioassay. Once the toxicity of the two fractions is determined, we can venture into ascertaining whether or not toxic compound(s) are present.

REFERENCES

1. Ranatunga, T. D.; Jervis, J. Helm, R. F. , Attachment to the Final Technical Report—Appendix C, NREL Subcontract XAC-4-13363-01, Reporting Period 31 October 1994-1 February 1997.

2. Kaar, W. E.; Lawrence, G.; Cool, M.; Merriman, M.; Brink, D. L. The Complete Analysis of Wood Polysaccharides using HPLC. *J. Wood Chem. Tech.* **11**(1991), 447-463.

3. Blumenkrantz, N.; Asboe-Hansen, G. New method for Quantitative Determination of Uronic Acids, *Anal. Biochem.* **54** (1973) 484-489.

4. Filisetti-Cozzi, T. M. C. C.; Carpita, N. C. Measurement of Uronic Acids Without Interference from Neutral Sugars, *Anal. Biochem.* **197** (1991) 157-162.